VOLUME 39 APRIL, 1960 NUMBER 4

# The Journal of Clinical Investigation

EDITED FOR THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION

## *Contents*

Turnover of Labeled Normal Gamma Globulin in Multiple Myeloma. STUART W. LIPPINCOTT, SAMUEL KORMAN, CONRAD FONG, ELMER STICKLEY, WILLIAM WOLINS AND WALTER L. HUGHES ..... 565

Some Effects of Restriction of Chest Cage Expansion on Pulmonary Function in Man: An Experimental Study. COLIN G. CARO, JOHN BUTLER AND ARTHUR B. DUBOIS ..... 573

Physiological Factors Affecting Airway Resistance in Normal Subjects and in Patients with Obstructive Respiratory Disease. JOHN BUTLER, COLIN G. CARO, RAPHAEL ALCALA AND ARTHUR B. DUBOIS ..... 584

Indocyanine Green: Observations on its Physical Properties, Plasma Decay, and Hepatic Extraction. GILBERT R. CHERRICK, SAMUEL W. STEIN, CARROLL M. LEEVY AND CHARLES S. DAVIDSON ..... 592

Studies on the Mechanism of the Plasma 17-Hydroxycorticosteroid Elevation Induced in Man by Estrogens. ELEANOR Z. WALLACE AND ANNE C. CARTER ..... 601

Free Fatty Acid Mobilization by Neuroadrenergic Stimulation in Man. JAMES T. HAMLIN, III, ROGER B. HICKLER AND ROBERT G. HOSKINS ..... 606

Hypocatalasemia: A New Genetic Carrier State. SHIGEO TAKAHARA, HOWARD B. HAMILTON, JAMES V. NEEL, THOMAS Y. KOBARA, YOSHIO OGURA AND EDWIN T. NISHIMURA ..... 610

Study of the Genetic and Extra-Genetic Determinants of Alpha-Ketosteroid Production in Man. ATTALLAH KAPPAS AND T. F. GALLAGHER ..... 620

Estimation of the Constancy of Density of the Fat-Free Body. EDWARD J. WERDEIN AND LAURENCE H. KYLE 626

The Effect of Neurohypophyseal Hormones on the Permeability of the Toad Bladder to Urea. ROY H. MAFFLY, RICHARD M. HAYS, EZRA LAMDIN AND ALEXANDER LEAF ..... 630

Studies on the Site of the Feedback Control of Cholesterol Synthesis. MARVIN D. SIPERSTEIN AND M. JOANNE GUEST ..... 642

Gastrodialysis in the Treatment of Acute Renal Failure. THOMAS A. MARR, JAMES M. BURNELL AND BELDING H. SCRIBNER ..... 653

Calcium and Phosphorus Homeostasis in the Parathyroidectomized Dog; Evaluation by Means of Ethylenediamine Tetraacetate and Calcium Tolerance Tests. PETER H. SANDERSON, FOSTER MARSHALL, II AND RICHARD E. WILSON ..... 662

Localization of Leucine Aminopeptidase in Serum and Body Fluids by Starch Gel Electrophoresis. O. DHODANAND KOWLESSAR, LORRAINE J. HAEFFNER AND MARVIN H. SLEISENGER ..... 671

Histamine Metabolism in Human Disease. GILDON N. BEALL AND PAUL P. VANARSDEL, JR. ..... 676

On the Mechanism of Impairment of Renal Concentrating Ability in Potassium Deficiency. ANDRZEJ MANITIUS, HOWARD LEVITIN, DAVID BECK AND FRANKLIN H. EPSTEIN ..... 684

On the Mechanism of Impairment of Renal Concentrating Ability in Hypercalcemia. ANDRZEJ MANITIUS, HOWARD LEVITIN, DAVID BECK AND FRANKLIN H. EPSTEIN ..... 693

Immunological Studies with Plasma Expanders Derived from Human Plasma. PAUL H. MAURER AND D. SUBRAHMANYAM ..... 698

Announcement of Meetings ..... 572

Program of the Fifty-Second Annual Meeting of the American Society for Clinical Investigation to be held in Atlantic City, N. J., May 2, 1960 ..... facing page 565

# The Journal of Clinical Investigation

Printed by the Lancaster Press, Inc., Prince and Lemon Sts., Lancaster, Pa. Published by the American Society for Clinical Investigation. Publication Office, 333 Cedar Street, New Haven 11, Conn.

---

PHILIP K. BONDY, EDITOR-IN-CHIEF

ASSOCIATE EDITORS

FRANKLIN H. EPSTEIN ALLAN V. N. GOODYER

**The Editorial Committee of the American Society for Clinical Investigation**

THOMAS P. ALMY, *New York, N. Y.*
SOLOMON A. BERSON, *New York, N. Y.*
GEORGE L. CURRAN, *St. Louis, Mo.*
LEWIS DEXTER, *Boston, Mass.*
I. S. EDELMAN, *San Francisco, Calif.*
CLEMENT A. FINCH, *Seattle, Wash.*
ALFRED P. FISHMAN, *New York, N. Y.*
ROBERT A. GOOD, *Minneapolis, Minn.*
JOHN B. HICKAM, *Indianapolis, Ind.*
ALEXANDER LEAF, *Boston, Mass.*
ARNOLD S. RELMAN, *Boston, Mass.*
I. HERBERT SCHEINBERG, *New York, N. Y.*
JOHN V. TAGGART, *New York, N. Y.*
RICHARD W. VILTER, *Cincinnati, O.*
KENNETH L. ZIERLER, *Baltimore, Md.*

---

THE JOURNAL OF CLINICAL INVESTIGATION is designed for the publication of original investigations dealing with or bearing on the problems of disease in man, and it is the policy of the editors that the JOURNAL should cover the field of clinical investigations in its broadest sense.

Contributions should be sent to the **Editor-in-Chief, Dr. Philip K. Bondy, 333 Cedar St., New Haven 11, Conn.** In order to hasten the process of reviewing and editing it is requested that each original manuscript be accompanied by a duplicate copy and duplicate illustrations.

In order further to expedite the earliest possible publication of articles submitted to the JOURNAL OF CLINICAL INVESTIGATION they will be published in the order of final processing after review, correcting and editing rather than in the strict sequence of their receipt in this office. Papers which require much editorial change, revision of charts and tables, condensation and rewriting will be delayed according to the time required for their correction. This policy will put a premium on excellence of manuscript as well as the major consideration of content. It is requested that figures be photographed, the photographs accompanying the manuscript. The originals of the figures need not be forwarded except by special request of the editors. For further instructions regarding the preparation of manuscripts, see the first page of the February, 1960, issue.

Each volume will consist of 12 monthly issues appearing throughout the calendar year. The subscription price is $15 per volume, **payable in advance**; foreign countries also $15, payable in U. S. funds. Single copies will be sold when available at Vols. 1–28, $2; Vols. 29–38, $1.50 a copy. Authors may purchase reprints at scheduled prices. **A special subscription rate of $6 per volume of 12 monthly issues is offered to medical students, interns, fellows, and residents of approved hospitals.** Subscriptions are **net postpaid.** Remittance should be made by draft, check or postal money order, payable to **The Journal of Clinical Investigation, 333 Cedar Street, New Haven 11, Conn.** Claims for copies lost in the mails must be received within **thirty** days (domestic), **ninety** days (foreign), of the date of issue. Changes of address must be received at least two weeks in advance of issue.

Second-class postage paid at Lancaster, Pa.

Copyright © 1960, American Society for Clinical Investigation

## COMMUNICABLE AND INFECTIOUS DISEASES

By FRANKLIN H. TOP, A.B., M.D., M.P.H., F.A.C.P., F.A.A.P., F.A.P.H.A.; with 21 collaborators. Just published. 1960, 4th edition, 812 pages, 6¼" x 9¼", 122 figures, 15 color plates. Price, $20.00.

## TEXTBOOK OF OTOLARYNGOLOGY

By DAVID D. DeWEESE, M.D.; and WILLIAM H. SAUNDERS, M.D. Just published. 1960, 464 pages, 6¼" x 9¼", 354 illustrations. Price, $8.75.

## PATHOLOGY

Edited by W. A. D. ANDERSON, M.A., M.D., F.A.C.P., F.C.A.P.; written by 35 pathologists. 1957, 3rd edition, 1402 pages, 6¼" x 10", 1294 illustrations, 11 color plates. Price, $16.00.

## OBSTETRICS AND GYNECOLOGY

By J. ROBERT WILLSON, M.D.; CLAYTON T. BEECHAM, M.D.; ISADOR FORMAN, M.D.; and ELSIE REID CARRINGTON, M.D. 1958, 605 pages, 6¼" x 9¼", 267 illustrations, Price, $10.75.

## REHABILITATION MEDICINE

By HOWARD A. RUSK, M.D. and 36 collaborators. 1958, 572 pages, 6¼" x 9¼", 172 figures. Price, $12.00.

## PHYSIOLOGY OF THE EYE

By FRANCIS HEED ADLER, M.A., M.D., F.A.C.S. New. 1959, 3rd edition, 794 pages, 6¼" x 9¼", 372 illustrations, including 2 in color. Price, $16.00.

## PRINCIPLES OF PUBLIC HEALTH ADMINISTRATION

By JOHN J. HANLON, M.S., M.D., M.P.H. Just published. 1960, 3rd edition, 714 pages, 6½" x 9½", illustrated. Price, $10.50.

## HANDBOOK OF ORTHOPAEDIC SURGERY

By ALFRED RIVES SHANDS, JR., B.A., M.D., in collaboration with RICHARD BEVERLY RANEY, B.A., M.D. 1957, 5th edition, 725 pages, 5¼" x 8¼", 214 illustrations. Price, $9.75.

*When you visit the*

**MOSBY**

*booth at your convention*

**Examine these new clinically oriented books**

## PRACTICE OF MEDICINE

Edited by JONATHAN CAMPBELL MEAKINS, C.B.E., M.D., LL.D., D.Sc. 1956, 6th edition, 1916 pages, 6¼" x 9¼", 318 illustrations, 4 in color. Price, $16.00.

## SURGICAL PATHOLOGY

By LAUREN V. ACKERMAN, M.D. In collaboration with HARVEY R. BUTCHER, JR., M.D. 1959, 2nd edition, 1096 pages, 6¼" x 9¼", 1114 illustrations. Price, $15.00.

## TEXTBOOK OF SURGERY

Edited by H. FRED MOSELEY, M.A., D.M., M.Ch. (Oxon), F.A.C.S., F.R.C.S. (Eng.), F.R.C.S. (C) New. 1959, 3rd edition, 1336 pages, 6¼" x 10", 738 text illustrations, 108 color plates, Price, $17.00.

## CLINICAL LABORATORY METHODS

By W. E. BRAY, B.A., M.D. 1957, 5th edition, 731 pages, 4¼" x 7¼", 124 illustrations, 18 color plates. Price, $9.75.

## PHYSICAL DIAGNOSIS

By JOHN A. PRIOR, M.D.; and JACK S. SILBERSTEIN, M.D. 1959, 388 pages, 6¼" x 9¼", 193 illustrations. Price, $7.50.

## MEDICAL PHYSIOLOGY

Edited by PHILIP BARD, with 13 contributors. 1956, 10th edition, 1421 pages, 6¼" x 9¼", 438 illustrations, 5 in color. Price, $14.00.

## LABORATORY MEDICINE—HEMATOLOGY

By JOHN B. MIALE, M.D. 1958, 735 pages, 6¼" x 9¼", 192 illustrations, 9 plates, 5 in color. Price, $13.75.

## HUMAN BIOCHEMISTRY

By ISRAEL S. KLEINER, Ph.D.; and JAMES M. ORTEN, Ph.D. 1958, 5th edition, 808 pages, 6¼" x 9¼", 94 text illustrations, 5 color plates. Price, $9.00.

*Gladly sent to teachers for consideration as textbooks.*

**The C. V. Mosby Company**
3207 Washington Boulevard • St. Louis 3, Missouri

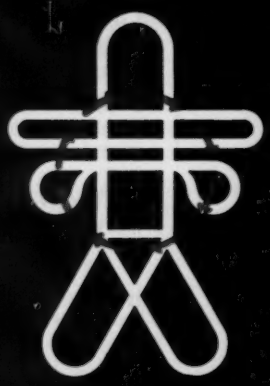
# DISSOLVES INTRAVASCULAR

## NOT JUST A NEW DRUG... *A NEW THERAPY*

# ACTASE

TRADEMARK

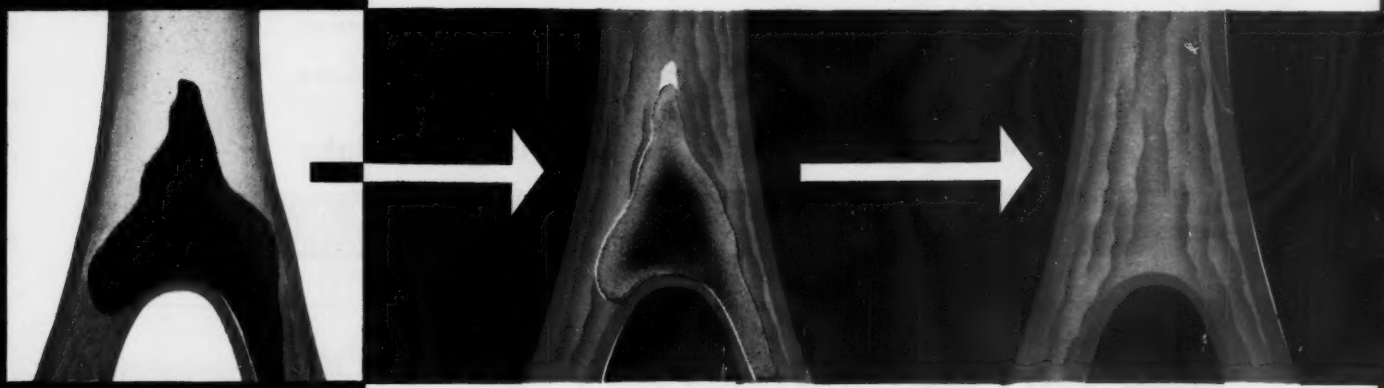
## Fibrinolysin (Human)




1. Moser, K.M.: J.A.M.A. *167*:1695 (Aug. 2) 1958.
2. Clifton, E.E.: J. Am. Geriatrics Soc. *6*:118, 1958.
3. Sussman, B.J., and Fitch, T.S.P.: J.A.M.A. *167*:1705 (Aug. 2) 1958.
4. Singher, H.O., and Chapple, R.V.: Clin. Med. *6*:439 (March) 1959.


ORTHO PHARMACEUTICAL CORPORATION, RARITAN, N. J.

# CLOTS in thrombophlebitis and pulmonary embolism



Clinically proved,[1-3] ACTASE has a specific lytic effect upon the venous thrombus or pulmonary embolus. Patients respond rapidly, often dramatically, to the clot-dissolving action of an intravenous infusion of this physiologic fibrinolysin.[4] A significant decrease in length of hospitalization following thrombophlebitis, as well as a reduction in the threat of pulmonary embolism, is now possible. In one series of patients with deep thrombophlebitis, some of whom had previously suffered pulmonary emboli, no occurrence of pulmonary emboli was reported following administration of ACTASE.[1]

Ortho

COMPLETE INFORMATION AVAILABLE ON REQUEST.

TOMORROW'S MEDICINE

**Books which crystallize today's avant-garde thinking into tomorrow's practice concepts**

## METAL-BINDING IN MEDICINE

**Proceedings of a Symposium Sponsored by Hahnemann Medical College and Hospital, Philadelphia**

*Marvin J. Seven, M.D., Editor. 61 Eminent Contributors.*

This is one of the first books to present a comprehensive collection of data on the uses of metal-binding agents in medicine. The contents of this volume begin with the basic concepts in trace metal research and continue with the clinical applications of some of the metal-binding agents as they have developed during the past ten years. Finally, several chapters explore some of the intriguing applications of the metal-binding principle in certain broad fields of clinical therapy.

400 PAGES 130 ILLUSTRATIONS NEW, 1960 $13.75

*IN PREPARATION:*

**Simon's ATTENUATED INFECTION: The Germ Theory in Contemporary Perspective**

**Sunderman & Sunderman's LIPIDS AND THE STEROID HORMONES IN CLINICAL MEDICINE**

**Order from your book store, or direct from the publisher**

J. B. LIPPINCOTT COMPANY
East Washington Square, Philadelphia 5, Pa.

NOW OVER 5100 BIOS CHEMICALS

- Ethyl Isothiocyanate
- Ethyl Lithium (1 Molar Solution)
- N-Ethylmaleimide
- Ethylmercuric Chloride
- Ethylmercuric Iodide
- Ethyl Nitrate
- 2-Ethylthiophene
- Etiocholenic Acid
- Europium
- Fabiatrin
- Ferrous Arsenate
- Ferrous Thiocyanate
- Ferulic Acid
- Ficin, cryst
- Fisetin
- Fluoboric Acid
- p-Fluoroaniline
- p-Fluorophenol
- Fluorotyrosine
- Forbisen
- p-Formylbenzoic Acid

*Ask for our new complete catalogue*

BIOS Laboratories, Inc.
17 West 60th St. New York 23 N. Y.
Plaza 7-8171

## WANTED

Back Issues of

THE JOURNAL OF CLINICAL INVESTIGATION

January, July, (Pt. I), September, through December, 1955

January through December, 1956

July and November, 1959

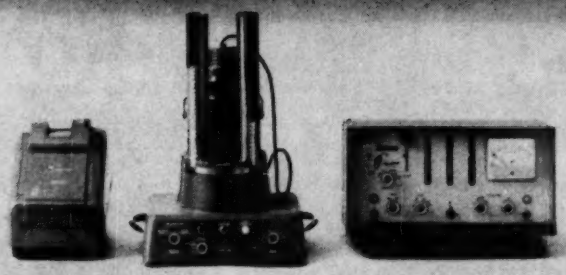
75¢ per copy and postage

*For particulars write to*

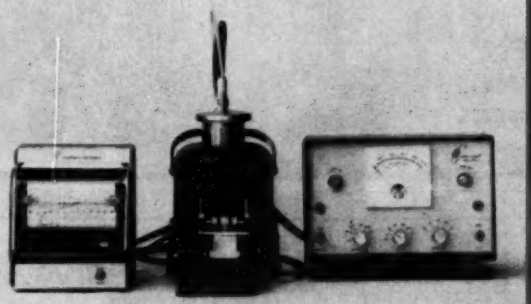
The Journal of Clinical Investigation
Business Office
333 Cedar Street
New Haven 11, Conn.

# PRECISION INSTRUMENTATION FOR NUCLEAR-MEASUREMENTS

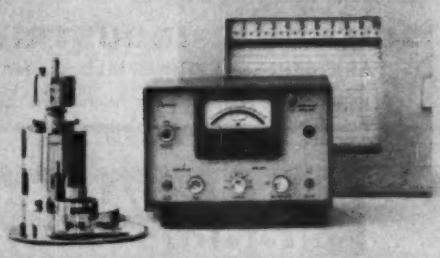
*The superbly designed systems for biological, clinical and industrial research shown here are part of the complete line of radioactivity measuring instruments developed and manufactured by Nuclear-Chicago. Instruments of superior quality, versatility and performance have made us the world's leading supplier to the nuclear industry. General Catalog R contains complete specifications and application information on these systems and details on our complete line. Please ask us for your copy.*



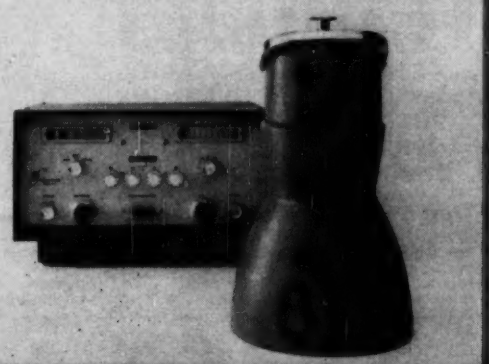
1



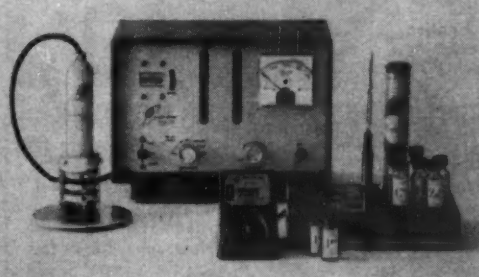
2



3



4



5

1. Automatic Sample Changer system accepts 35 radioactive samples and presents printed data on length of time required to accumulate predetermined number of counts.
2. Actigraph® system presents record of position and total activity of radioactive spots on strip chromatograms.
3. The Dynacon® Electrometer measures soft beta samples in solid, liquid, or gaseous phases. Ideal for continuous flow analysis.
4. Scintillation Well Counter and Scaler-Spectrometer permit low background measurements of gamma emitting liquid or solid samples.
5. Model 4000 Training System includes scaler, timer, G-M Tube, sample mount, radioactive reagents, pipette, sample pans.

nuclear-chicago CORPORATION

329 HOWARD AVE. · DES PLAINES, ILL.

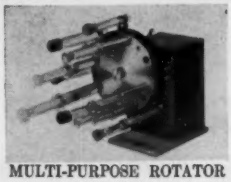
LEON KAPELSOHN

**"I'll Be Looking for you at Booth 42 on the Steel Pier at Atlantic City May 1–3"**

You'll see new devices for faster, better testing procedures such as our new syringe attachments for pipetting, our new pH meter, and our new Vortex Mixer.
You'll see the newest models of *old reliable* instruments for micro-chemical clinical testing.
You'll see some surprises and get information about still newer ideas being developed.

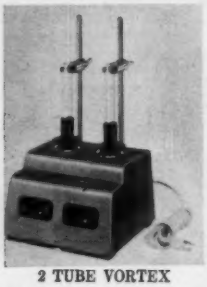
- NATELSON MICROGASOMETER
- SCIENTIFIC INDUSTRIES ULTRA-BURET
- BLOOD COLLECTING TUBES with PLASTIC CLOSURES
- MULTI-PURPOSE ROTATOR
- LABORATORY TIMERS
- AUTOMATIC SYRINGE ATTACHMENT
- VORTEX TEST TUBE MIXER
- NATELSON pH METER

**SCIENTIFIC INDUSTRIES, INC.**
**15 PARK STREET, SPRINGFIELD 3, MASSACHUSETTS**
*Manufacturers of Laboratory Instruments and Equipment*



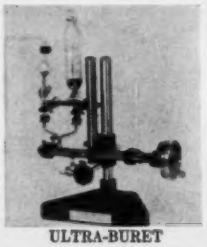
MULTI-PURPOSE ROTATOR

NATELSON MICROGASOMETER



2 TUBE VORTEX

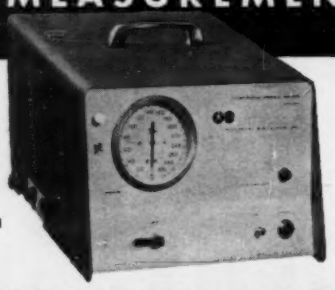
4 TUBE VORTEX



ULTRA-BURET

SYRINGE ATTACHMENT

**NEW ADVANCE IN BLOOD PRESSURE MEASUREMENT**



American Society for Clinical Investigation Meeting Booth 33

**CONTINUOUS SYSTOLIC MONITOR**

- Accurate *continuous* measurement of blood pressure
- No arterial puncture—adult, pediatric and animal application
- Measures response to emotional and external stimuli
- Manometer pressure gauge plus connection for auxiliary recorder
- For pharmacological studies, pediatric research, operating and recovery rooms.

Write for Brochure CSM-2 or Demonstration
Representatives throughout U.S. and Canada

BIOPHYSICAL ELECTRONICS, INC.
NEW HOPE, PA.

**RARE and FINE ORGANICS**

A few of the thousands of rare stock chemicals

o-AMINOBIPHENYL
N-ETHYL MALEIMIDE
d-GLUCURONIC ACID
5-HYDROXYINDOLE-3-ACETIC ACID
5-HYDROXYTRYPTOPHANE
IMIDAZOLE ACETIC ACID
α-KETOISOCAPROIC ACID
α-KETOISOVALERIC ACID
β-MERCAPTOETHYLAMINE
PHLORETIN
o-PHTHALALDEHYDE
SEROTONIN (Creatinine Sulfate)

**WRITE FOR CATALOG #3 LISTING OVER 12,000 RARE—**

| | | |
|---|---|---|
| alkaloids | hydrocarbons | steroids |
| amino acids | keto acids | sugars |
| enzymes | Fatty acids | purines |
| biochem preps | peptides | carcinogens |

*Write Dept. "C"*

**K & K LABORATORIES, INC.**
177-10 93rd AVENUE JAMAICA 33, N Y

# *Leukemia.....*

## 'LEUKERAN'® brand

### CHLORAMBUCIL (formerly known as C. B. 1348)

**FOR CHRONIC LYMPHOCYTIC LEUKEMIA**

A derivative of nitrogen mustard, it has provided amelioration of follicular lymphoma, lymphocytic lymphoma with or without leukemia, and Hodgkin's disease.

**Sugar-coated Tablets of 2 mg.**

## 'MYLERAN'® brand

### BUSULFAN

**FOR CHRONIC MYELOCYTIC LEUKEMIA**

'Myleran' has been reported to induce remissions, lasting up to two years, in chronic myelocytic leukemia. In addition to the decrease in total white cell count and a selective reduction of immature myeloid cells, it usually gives, early after its administration, a rise in hemoglobin level and pronounced subjective improvement.

**Tablets of 2 mg.**

## 'PURINETHOL'® brand

### MERCAPTOPURINE

**FOR ACUTE LEUKEMIA AND CHRONIC MYELOCYTIC LEUKEMIA**

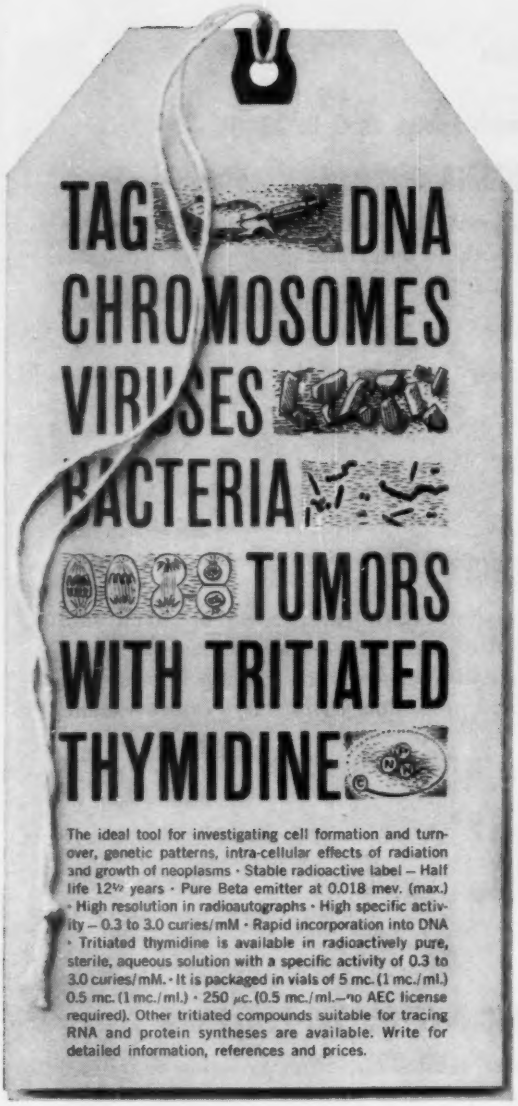
'Purinethol' provides worth-while temporary remissions, either partial or complete, in a high percentage of patients. In general, a higher proportion of children than adults with acute leukemia respond favorably.

**Tablets of 50 mg.**

Facilities for complete and frequent blood counts must be available for patients receiving 'Leukeran', 'Myleran' or 'Purinethol'.

Full information about these products will be sent on request.

**BURROUGHS WELLCOME & CO. (U.S.A.) INC.,** Tuckahoe, New York


TAG DNA
CHROMOSOMES
VIRUSES
BACTERIA
TUMORS
WITH TRITIATED
THYMIDINE
The ideal tool for investigating cell formation and turnover, genetic patterns, intra-cellular effects of radiation and growth of neoplasms · Stable radioactive label – Half life 12½ years · Pure Beta emitter at 0.018 mev. (max.) · High resolution in radioautographs · High specific activity – 0.3 to 3.0 curies/mM · Rapid incorporation into DNA · Tritiated thymidine is available in radioactively pure, sterile, aqueous solution with a specific activity of 0.3 to 3.0 curies/mM. · It is packaged in vials of 5 mc. (1 mc./ml.) 0.5 mc. (1 mc./ml.) · 250 μc. (0.5 mc./ml.—no AEC license required). Other tritiated compounds suitable for tracing RNA and protein syntheses are available. Write for detailed information, references and prices.

SCHWARZ BIORESEARCH, INC.
Dept. DG · Mount Vernon, New York
BIOCHEMICALS · RADIOCHEMICALS · PHARMACEUTICALS
*for research, for medicine, for industry*

PROTECT FEED DOLLARS WITH
PURINA HEALTH AIDS

# KILL GERMS
with
## PURINA DISINFECTANT

Solution costs only a penny a gallon! . . . at your
**PURINA DEALER'S**

## J.C.I. SUPPLEMENTS

*Order while still available*

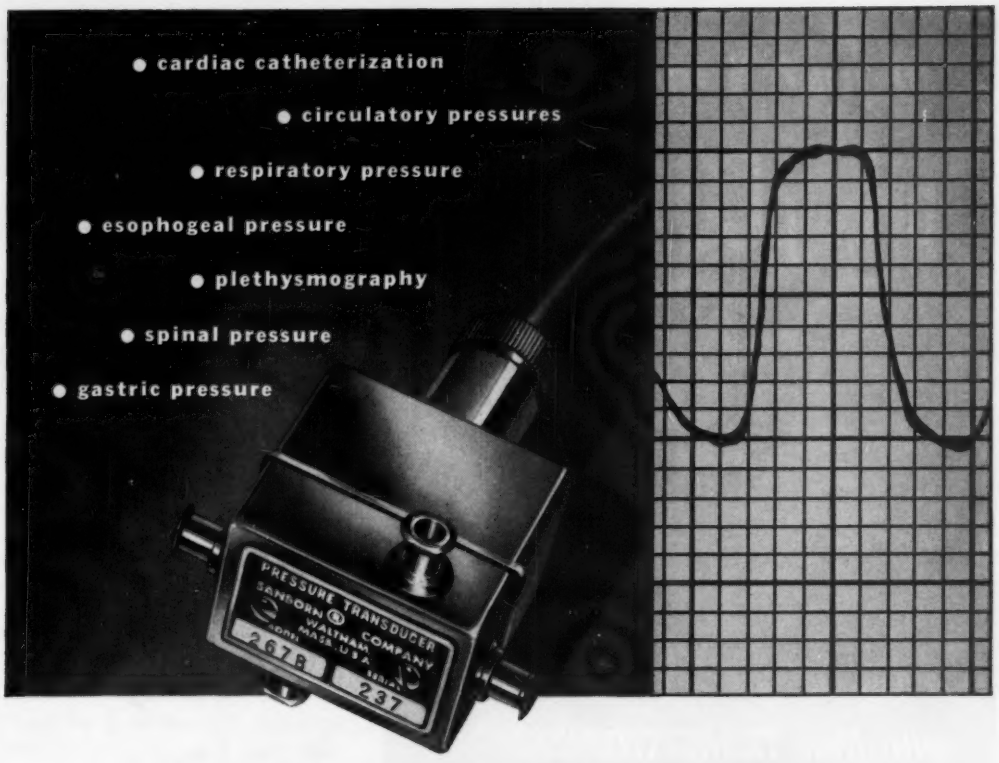
| | |
|---|---|
| Symposium on Malaria<br>May, 1948, Part II | $2.00 |
| Symposium on Antibiotics<br>September, 1949, Part I | $2.00 |
| Symposium on Radioactive Isotopes<br>November, 1949, Part I | $2.00 |
| Treatment of Acute Infectious Hepatitis<br>July, 1955, Part II | $1.50 |
| The Renal Lesion in Epidemic Hemorrhagic Fever<br>January, 1957, Part II | $1.50 |
| Metabolic Studies in Paralytic Acute Anterior Poliomyelitis<br>June, 1957, Part II | $1.50 |

* * * * *

| | |
|---|---|
| Studies on Influenza in the 1957–1958 Pandemic<br>January, 1959, Part II | $1.50 |
| History of the A.S.C.I.<br>July, 1959, Pt. II | $1.50 |

*Order through the Business Office of*

**THE JOURNAL OF CLINICAL INVESTIGATION**
333 Cedar Street New Haven 11, Conn.




## *for* SINGLE *and* DIFFERENTIAL PRESSURE MEASUREMENTS, SANBORN TRANSDUCERS *provide*

### CHOICE OF SENSITIVITIES

Sanborn Physiologic Pressure Transducers, in two sensitivity series — 1 cm/mm Hg or 1 cm/0.1 mm Hg — provide models for both differential (difference between two variable pressures) and single-ended (difference between a variable pressure and atmospheric pressure) measurement.

**Compact, Precision Construction**

Durable, non-corrosive Monel construction resists normal saline, alcohol and other standard physiologic and sterilization solutions. Chamber design permits flushing liquid to sweep the entire pressure chamber, removing all bubbles. Standard luer female type connections accept all needle and catheter fittings. Extremely small size (1½" high x 1 9/16" wide x 1" deep) allows transducers to be easily supported by ring stand clamp or similar method for use close to the subject under test.

| | Sensitivity | nominal working range | volume displacement |
|---|---|---|---|
| Model 267 | 1 cm/1 mm Hg | −100 to +400 mm Hg | 0.05 mm³/100 mm Hg |
| Model 268 | 1 cm/0.1 mm Hg | −40 to +40 mm Hg | 0.5 mm³/100 mm Hg |

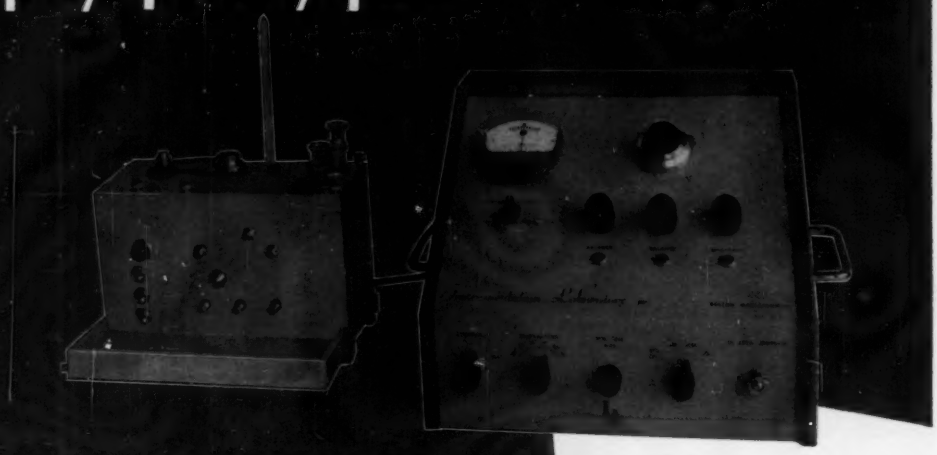
**Complete data on the pressure transducers, as well as compatible amplifying, monitoring and recording instruments, is available in a new 42-page "Biophysical Instrument" catalog. Copies are available on request from Inquiry Director, Waltham, Mass.**

# SANBORN COMPANY

Medical Division • 175 Wyman Street, Waltham 54, Mass.

# *in just 1 minute each*

# pH, $pCO_2$, $pO_2$ *measurements in blood*



*with New, TRANSISTORIZED*

# I. L. METER

*for maximum accuracy*

• ±.002pH units • ±.25% $pO_2$ • ±1% $pCO_2$

See the I. L. Meter in operation! BOOTH 16 American Society for Clinical Investigation Meeting Atlantic City — May, 1960

Developed to complement the $pCO_2$ and $pO_2$ electrodes designed by Dr. John Severinghaus, the I. L. Meter insures dependability and accuracy up to the full capacity of the electrode system.

The meter, a compact, chopper stabilized, transistorized, D.C. amplifier with accompanying bath, is engineered to give years of outstanding performance. The unit is currently in use at leading hospitals and laboratories.

A uniquely designed four-position switch system, enables the desired electrodes to be selected instantly. This same system enables the mercury batteries to be calibrated against a Weston standard cell, making possible a check and correction for the zero drift of the unit at any time. The entire procedure is accomplished without changing the balance attained by a standard buffer. An indicator for checking the membrane on the $pCO_2$ and $pO_2$ electrodes is provided.

**FREE TRIAL PERIOD:** The I. L. Meter is available to you on a 10-day trial period without cost or obligation.

I. L. Meter is guaranteed for one year, serviced by factory trained personnel.

Write for free, comprehensive brochure

**INSTRUMENTATION LABORATORY, INC.**

Dept. E, 121 Jersey Street, Boston, Massachusetts or —
Medical Division, National Welding Co., 218 Fremont, San Francisco, Calif.

# *The Blakiston Division invites you* to visit Booth 34 and examine its outstanding medical publications

**Principles of Biochemistry, Second Edition**

**By A. White, Ph.D.; E. L. Smith, Ph.D.; D. Stetten, Jr., M.D., Ph.D.; 1149 pp., 6 x 9, illus., $15.00**

This new second edition is an exceptionally modern, well-balanced presentation, emphasizing the fundamental principles and concepts of biochemistry. As a result of important new advances in the field of metabolism and endocrinology, these chapters have been completely rewritten.

**Manual of Histologic and Special Staining Techniques, Second Edition**

**Armed Forces Institute of Pathology**

**Edited by Lawrence P. Ambrogi; 8 x 10½, illus., $5.50**

This manual presents basic techniques in the most simplified and direct manner commensurate with good results. Special staining techniques have been adopted to use with routinely fixed tissues. Alternative methods are given in many instances so that laboratories lacking the complete reagent stock of the Institute can choose methods for which they are equipped.

**Fundamentals of Modern Allergy**

**Edited by Samuel J. Prigal, M.D., F.A.C.P.; Sponsored by the New York Allergy Society with 58 contributors; 690 pp., 125 illus., 6 x 9, $18.50**

This contributed volume makes available to non-specialists as well as allergists basic information on allergy with emphasis on diagnosis and treatment. Theoretical aspects are also given consideration since this will provide a better understanding of the mechanisms of allergic diseases. This volume is truly modern in that newer areas of allergy are introduced for the first time in a text.

**Electrophysiology of the Heart**

**By Brian F. Hoffman, M.D.; Paul F. Cranefield, Ph.D.; 5⅝ x 8⅜, illus., $12.50**

A comprehensive work on the electrophysiology of the heart, this book summarizes the important information available and adds much new material in this very interesting and rapidly developing field. This work summarizes the results of ten years research on the technique of single-cell recording and relates them to clinical techniques and findings

**Bone as a Tissue**

**Edited by Kaare Rodahl, M.D.; Jesse T. Nicholson, M.D.; Ernest M. Brown, Jr., M.D.; 358 pp., 6 x 9, illus., $16.00**

Presenting a review of the osseous system and of the significant advances in recent years, this work correlates and integrates basic research information into clinical medicine. Each chapter is written by an internationally known authority. The material is divided into four general subject areas: Osteoporosis; Dynamics of Calcium Metabolism; Ultrastructure of Bone; and Vitamin D, Parathyroids, Citric Acid, Calcium, and Phosphorus.

**The Heinz Handbook of Nutrition**

**Edited by Benjamin T. Burton, Ph.D.; 447 pp., 6 x 9, illus., $5.75**

This definitive manual condenses and encompasses the entire field of human nutrition, both in health and in disease; and represents the accepted consensus of contemporary scientific and clinical thought summarizing up-to-date, proven and accepted information.

---

## Blakiston Division, McGraw-Hill Book Co.

**330 W. 42nd St., New York 36, N. Y.—68 Post St., San Francisco 4, Calif.**

*Simplicity...*
*Dependability*

# Transaminase Kits

*by* DADE

**Accurate colorimetric method for determination of SGO-T and SGP-T**

Transaminase Kits provide reliable and reproducible results. Each kit contains reagents for 100 tests. Calibration standard is in liquid form and maintains its stability indefinitely. Conveniently packaged so reagents may be kept together when not in use.

***SIMPLE PROCEDURE FOR DETERMINATION OF SGO-T AND SGP-T***

Add 0.2 ml of serum to prepared buffered substrate and incubate in 37°C. water bath for one hour. (Only 30 minutes required for SGP-T.) Add color developer. Allow to stand at room temperature for 20 minutes. Add 10 ml 0.4N NaOH to tube and allow to stand for 5 minutes. (Sodium hydroxide not supplied with kit.) Read in any laboratory colorimeter at 490 to 510 $m\mu$ wavelength.

No. 53951—Transaminase Kit for SGO-T, complete with Aspartic-Keto substrate, DNPH color developer and calibration standard ..... $12.50

No. 53952—Transaminase Kit for SGP-T, complete with Alanine-Keto substrate, DNPH color developer and calibration standard ..... $12.50

**Scientific Products**

DIVISION OF AMERICAN HOSPITAL SUPPLY CORPORATION

GENERAL OFFICES—1210 LEON PLACE, EVANSTON, ILLINOIS

Regional Offices: Atlanta · Boston · Chicago · Columbus · Dallas · Kansas City · Los Angeles
Minneapolis · New York · San Francisco · Washington

CLEANEST CLEANEST CLEANEST CLEANEST CLEANEST CLEANEST CLEAN

# THE WORLD'S FINEST DETERGENT

# ALCONOX

***Made especially for the exacting requirements of LABORATORY, HOSPITAL AND MEDICAL USE!***

**MORE EFFECTIVE than any known detergent in powder form or any liquid detergent that costs four times as much!**

**Also makers of ALCOJET for all equipment washed by machine and ALCOTABS in tablet form for all pipette washers.**

***Order from your supplier or ask him for samples and FREE Cleaning Guide.***

**Meets Highest Government Specifications**

**Sold by All Leading Laboratory, Hospital and Surgical Supply Dealers in the United States as well as in**

**CANADA • ENGLAND • SWITZERLAND • BELGIUM • NETHERLANDS • MEXICO VENEZUELA • PERU • BRAZIL • PANAMA • PUERTO RICO • HAWAII and BELGIAN CONGO**

**ALCONOX, INC., 853 BROADWAY, NEW YORK 3, N.Y.**

## *CURRENT COMMENTS:*

Important facts concerning "Factor VII contamination" and the general reliability of thromboplastin preparations have been collected and compiled for practical use in your laboratory. Based on experiments and extensive testing, this information clarifies many misconceptions regarding thromboplastins and their influence over accuracy in prothrombin time testing. For your free copies of the *Current Comments* series write to Laboratory Supply Division Warner-Chilcott, Morris Plains, N. J.

It is the essence of common sense that reagents used in vital determinations should give the same answer from bottle to bottle, in a single box, on the same patient's specimen. It is amazing that many thromboplastin products sold to the laboratories do not meet this most essential requirement.

Check the uniformity of your thromboplastin by a simple three-vial test.

Reconstitute three vials of Simplastin and any other thromboplastin. Use a plasma from a patient in the therapeutic range having a prothrombin activity less than 20 per cent of normal (over 30 seconds). Compare the results for yourself. Trial Simplastin for this comparison will be sent on your request.

**Simplastin®**

# PROGRAM OF THE FIFTY-SECOND ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION TO BE HELD IN ATLANTIC CITY, NEW JERSEY, MAY 2, 1960

1. The Induction of Heterologous Immunity to Influenza by Aerosol Administration of Inactivated Virus. JEROME L. SCHULMAN and EDWIN D. KILBOURNE, New York, N. Y.
2. On the Role of Virus Sulfhydryl Groups in the Attachment of Enteroviruses to Cells. LENNART PHILIPSON and PURNELL W. CHOPPIN, New York, N. Y.
3. The Capacity of Tubercle Bacilli to Assume a Latent State *In Vivo*. ROBERT M. MCCUNE, New York, N. Y.
4. Studies on the Mechanism of Fever Following Intravenous Inoculation of Staphylococci. ELISHA ATKINS and LAWRENCE R. FREEDMAN, New Haven, Conn.
5. Relationship of Phagocytosis to the Fall in CSF Glucose in Experimental Meningitis. ROBERT G. PETERSDORF, Baltimore, Md. and Seattle, Wash.
6. Studies of Granulocyte Kinetics. JOHN W. ATHENS, ALVIN M. MAUER, SPENCER O. RAAB, OTTO P. HAAB and GEORGE E. CARTWRIGHT, Salt Lake City, Utah.
7. Presence of an Altered Amino Acid Sequence in Hgb M (Boston Type). PARK S. GERALD, MARY L. EFRON and MARGARET J. PEASE, Boston, Mass.
8. Oxidative Hemolysis and Precipitation of Hemoglobin: Heinz Body Anemias as an Accelerated Form of Red Cell Aging. JAMES H. JANDL and DAVID W. ALLEN, Boston, Mass.
9. The Enzymatic Defect of Orotic Aciduria. LLOYD H. SMITH, JR. and CHARLES M. HUGULEY, JR., Boston, Mass. and Atlanta, Ga.
10. Relation of Hormone-Receptor Bonding and Antidiuretic Action of Vasopressin. IRVING L. SCHWARTZ, HOWARD RASMUSSEN, MARY ANNE SCHOESSLER, CONRAD T. O. FONG and LAWRENCE SILVER, Upton, N. Y.
11. Micropuncture Study of Net Transtubular Movement of Water and Urea in the Rat Kidney. WILLIAM E. LASSITER, CARL W. GOTTSCHALK and MARGARET MYLLE, Chapel Hill, N. C.
12. Effect of Acute Uremia on Arterial Blood Pressure in Rabbits. CARMELO GIORDANO, HERMAN WEINSTOK and JOHN P. MERRILL, Boston, Mass.
13. A "Precursor Solution" of Pancreatic Juice: Evidence for an Exchange Mechanism. H. D. JANOWITZ and D. A. DREILING, New York, N. Y.
14. The Effect of Wheat Instillation into the Proximal Ileum of Patients with Idiopathic Sprue. CYRUS E. RUBIN, LLOYD L. BRANDBORG, ARNOLD L. FLICK, CHERILL PARMENTIER, PATRICIA PHELPS and SALLY VAN NIEL, Seattle, Wash.
15. The Effect of Changes in Atrial Systole on the Relation Between Mean Atrial Pressure and Stroke Work. STANLEY J. SARNOFF, JERE H. MITCHELL and JOSEPH P. GILMORE, Bethesda, Md.
16. Metabolic Regulation of Cardiac Output. WILLIAM E. HUCKABEE, Boston, Mass.
17. On the Mechanism of Carotid Sinus Function. LYSLE H. PETERSON, ERIC O. FEIGL and PETER COURAS, Philadelphia, Pa.
18. Effect of Antihypertensive Treatment in the Rat on the Potentiation of Atherogenesis by Experimental Hypertension. QUENTIN B. DEMING, MARIE M. DALY, JAIME BLOOM, LILI BRUN and RUTH KAPLAN, New York, N. Y.
19. Chromosome Constitution in Human Gonadal Disorders. AVERY A. SANDBERG, THEODORE S. HAUSCHKA, EDWIN GORDY and GEORGE F. KOEPF, Buffalo, N. Y.
20. Transmanganin, the Specific Manganese-Carrying Protein of Human Plasma. GEORGE C. COTZIAS and ALBERT J. BERTINCHAMPS. Upton, N. Y.
21. Inhibition of Aromatic Amino Acid Decarboxylation in Man and Associated Pharmacologic Effects. JOHN A. OATES, JR., LOUIS GILLESPIE, JR., J. RICHARD CROUT and ALBERT SJOERDSMA, Bethesda, Md.
22. Immunoassay of Plasma Insulin Concentrations in Normal and Diabetic Man: Insulin Secretory Response to Glucose and Other Agents. ROSALYN S. YALOW and SOLOMON A. BERSON, Bronx, N. Y.
23. The Krebs Cycle and Diabetic Ketosis. DANIEL W. FOSTER and MARVIN D. SIPERSTEIN, Dallas, Tex.
24. Mannose Metabolism, an Index of Glucose Utilization. GEORGE F. CAHILL, JR., FRANCIS C. WOOD, JR., BERNARD LEBOEUF and ALBERT E. RENOLD, Boston, Mass.
25. Stimulation of Hepatic Fatty Acid Synthesis by Ethanol *In Vivo* and *In Vitro*. CHARLES S. LIEBER, LEONORE M. DECARLI and RUDI SCHMID, Boston, Mass.
26. The Effect of Cell Structure and Growth Hormone on Protein Synthesis in Striated Muscle. DAVID M. KIPNIS and ERIC REISS, St. Louis, Mo.

# LISTING OF EXHIBITS AT FIFTY-SECOND ANNUAL MEETING OF THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION, 1960

## SCIENTIFIC EXHIBIT

### AN ELECTRIC CIRCUIT FOR IMPROVING THE DYNAMIC RESPONSE OF THE CONVENTIONAL CARDIAC CATHETER SYSTEM

by


FRANK W. NOBLE

*Laboratory of Technical Development, National Heart Institute, Bethesda Md.*

and

GUY BARNETT

*Laboratory of General Medicine and Experimental Therapeutics, National Heart Institute, Bethesda, Md.*


## TECHNICAL EXHIBITS

| | | | |
|---|---|---|---|
| ABBOTT LABORATORIES | #38 | LEA & FEBIGER | #10 |
| ADVANCED INSTRUMENTS, INC. | #8 | E. LEITZ, INC.* | #30 |
| ATOMIC ASSOCIATES INC.* | #29 | J. B. LIPPINCOTT COMPANY* | #26 |
| BAUSCH & LOMB OPTICAL CO. | #45 | LITTLE, BROWN AND COMPANY | #2 |
| BIOPHYSICAL ELECTRONICS, INC.* | #33 | THE MACMILLAN COMPANY | #51 |
| BLAKISTON DIVISION, McGRAW-HILL BOOK CO., INC.* | #34 | THE C. V. MOSBY COMPANY* | #22 |
| CAMBRIDGE INSTRUMENT COMPANY, INC. | #3 & #4 | NUCLEAR-CHICAGO CORPORATION* | #31 & #32 |
| COULTER ELECTRONICS, INC.* | #1 | PACKARD INSTRUMENT COMPANY, INC.* | #25 |
| CUSTOM ENGINEERING & DEVELOPMENT COMPANY* | #6 | PHIPPS & BIRD, INC. | #39 |
| E-C APPARATUS COMPANY | #15 | PHOENIX PRECISION INSTRUMENT CO., INC.* | #50 |
| ELECTRONICS FOR MEDICINE, INC. | #35 & #36 | PICKER X-RAY CORPORATION | #7 |
| GILFORD INSTRUMENT LABORATORIES | #37 | SANBORN COMPANY* | #27 & #28 |
| GRUNE & STRATTON, INC. | #23 | W. B. SAUNDERS COMPANY | #40 & #41 |
| PAUL B. HOEBER, INC. | #43 | SCIENTIFIC INDUSTRIES, INC.* | #42 |
| INSTRUMENTATION ASSOCIATES* | #20 | IVAN SORVALL, INC. | #24 |
| INSTRUMENTATION LABORATORY, INC.* | #16 | E. R. SQUIBB & SONS | #9 |
| | | THE WATERS CORPORATION | #44 |

THE COCA-COLA COMPANY
will serve in exhibit area

* See advertisement in this issue.

# TURNOVER OF LABELED NORMAL GAMMA GLOBULIN IN MULTIPLE MYELOMA *

By STUART W. LIPPINCOTT, SAMUEL KORMAN,† CONRAD FONG, ELMER STICKLEY, WILLIAM WOLINS AND WALTER L. HUGHES

*(From the Medical Department, Brookhaven National Laboratory, Upton, N. Y.)*

(Submitted for publication September 16, 1958; accepted December 4, 1959)

Aberrations of protein metabolism in multiple myeloma have been studied by a number of investigators (1–6) to ascertain whether the globulins in the blood consist of excesses of normal globulins or of chemically abnormal globulins, or both. Such research has been directed toward determining whether "characteristic protein abnormalities" could be distinguished by *1*) separation of the serum proteins with identification of their physicochemical properties by using the Tiselius electrophoresis apparatus and the analytical ultracentrifuge; *2*) certain structural relationships indicated by the amino end-group method; *3*) immunochemical procedures; and *4*) detection of formation of abnormal protein by administering to the patient isotopically labeled amino acids, the tracers being the stable isotopes $N^{15}$ and $C^{13}$ or radioactive carbon, $C^{14}$. By use of such experimental methods it has been reported that at least some, if not all, of the quantitatively excessive globulins produced in this disease are abnormal.

Our approach to the investigation of certain fundamental aspects of protein metabolism in this disease has been concerned with the patient's turnover of globulins. Data are being presented which were obtained by determining the biological half-life of gamma globulin fractionated from normal individuals, labeled with $I^{131}$ and injected into ten patients with clinically proven multiple myeloma. A subsequent report will be devoted to the findings when the source of the fractionated gamma globulin was from patients with multiple myeloma. The latter study permitted observations on the half-life of labeled autologous and homologous preparations in this disease.

The electrophoretic pattern of the serum of each patient was determined to establish the presence of a predominant and characteristic peak. It has been stated previously by others (*7*–*9*) that a sharply defined peak is due to the presence of large amounts of anomalous protein components. Because of the similarity in mobility of these peaks to those of the corresponding normal serum protein components, the peaks have been designated by the normal appellation. Six categories have been reported in this disease: alpha type, beta type, gamma type, M peak migrating between the gamma and beta peaks, multiple peaks, and minor anomalies. It has been categorically stated in some of the literature referred to above that for a given case the abnormal protein retains the same electrophoretic mobility throughout the course of the patient's disease. Data substantiating this statement are difficult to find, but there is one study (10) in which the protein distribution was followed by electrophoresis for one to three years in six patients with this disease. In four of these cases there was a persistence of the same peak with time, and in each instance there was with time an increase in the amount of the anomalous protein. In two other cases in this group, the final well established peak was hardly noticeable earlier in the course of the disease, but with time it became prominent. For our purposes the patients' electrophoretic diagram before the time of injection of the labeled normal gamma globulin was determined. These patients are shown in Figures 1 A and B and include in some instances patterns made about three to six months apart. The half-life of the individual globulin turnover has been considered both separately and with reference to the protein distribution in the serum as indicated by the electrophoretic pattern.

## PATIENTS AND METHODS

*Source and preparation of normal gamma globulin which was injected after labeling.* The gamma globu-

* This research was supported by the United States Atomic Energy Commission.

† Research Collaborator from the Montefiore Hospital, New York, N. Y.

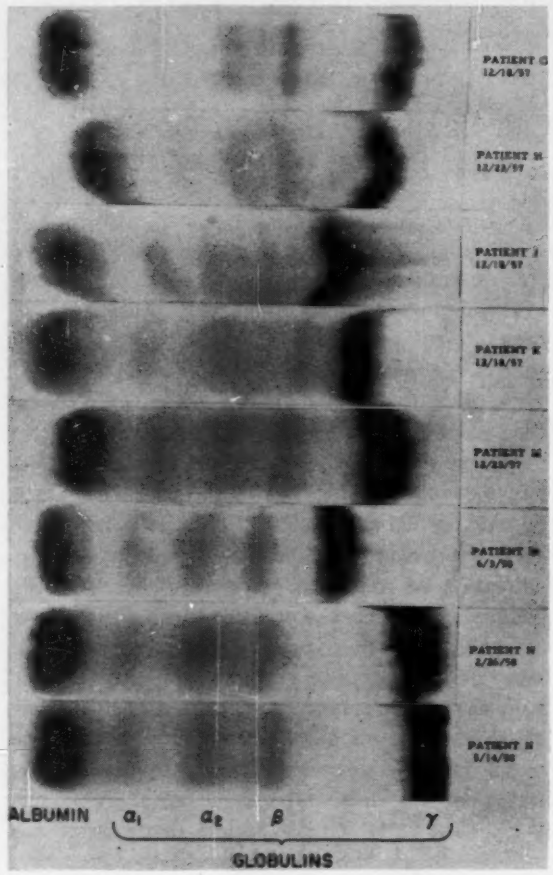



FIG. 1A. SERUM PAPER ELECTROPHORETIC PATTERN WITH GAMMA PEAK IN PATIENTS WITH MULTIPLE MYELOMA.

lins used for turnover studies were prepared from individual serums by the method of Nichol and Deutsch (11); the serum was diluted with 3 vol of cold distilled water and adjusted to pH 7.7 by the addition of 0.05 M $CH_3$-COOH or $Na_2HPO_4$, 50 per cent ethanol was then added slowly with stirring to a final concentration of 20 per cent (vol/vol), the temperature being simultaneously decreased to $-5°$ C. After standing about an hour, the precipitate was centrifuged and resuspended in 10 to 20 vol of water at 0° C and the pH adjusted to 5.1 by the addition of 0.05 M acetic acid. Fifty per cent ethanol was then slowly added to 0° C to give a final concentration of 15 per cent and after standing about an hour, the precipitate was centrifuged off and stored at $-20°$ C for possible future fractionation. The supernatant was adjusted to pH 5.6 by the addition of 0.05 M $Na_2HPO_4$ and 50 per cent ethanol was added to a final concentration of 12 per cent at $-3.5°$ C. This precipitate was also removed and stored for possible future use. The gamma globulin remaining in the supernatant was precipitated by adjusting the pH to 7.3 by the addition of 0.05 M $Na_2HPO_4$ followed by the addition of 50 per cent ethanol to a final concentration of 25 per cent. After centrifuging, the precipitated gamma globulin (ppt. C-2-$\gamma_2$ globulin by the designation of Nichol and Deutsch) was suspended and dialyzed against distilled water overnight and dried from the frozen state. The yield approximated 400 mg per 100 ml of normal serum. The products contained only gamma globulin as judged by electrophoretic (Tiselius) analysis.

The electrophoretic characterization of this chemical fraction was carried out in the commonly used pH 8.6 barbital buffer in a standard Tiselius apparatus at a bath temperature of 0° C, a field strength of 8.9 volts per cm, and a protein concentration of about 1 per cent. After variable periods of runs up to 180 minutes, the patterns were recorded by the Longsworth scanning method. In each of the preparations used for labeling, more than 95 per cent of the protein traveled in a single Gaussian envelope with the mobility of gamma globulin. Before administration each preparation was passed through Seitz filters and cultured for sterility. Two donor sources, a 34 year old female and a 35 year old male, provided the blood for fractionation. Each was an asymptomatic volunteer, hospitalized and, after being extensively studied, considered as a suitable healthy donor. The establishment of "normality" was considered important because in subsequent studies the globulins were prepared from patients with multiple myeloma and thus could differ chemically or structurally.

*Labeling of gamma globulin with radioactive iodine.* Iodination was carried out following the principles established for the iodination of serum albumin by Hughes and Straessle (12); 100 to 150 mg of gamma globulin was dissolved in 1 to 2 ml of 0.01 N $NH_4OH$ at 0° C. The iodinating reagent was prepared by adding 1 drop

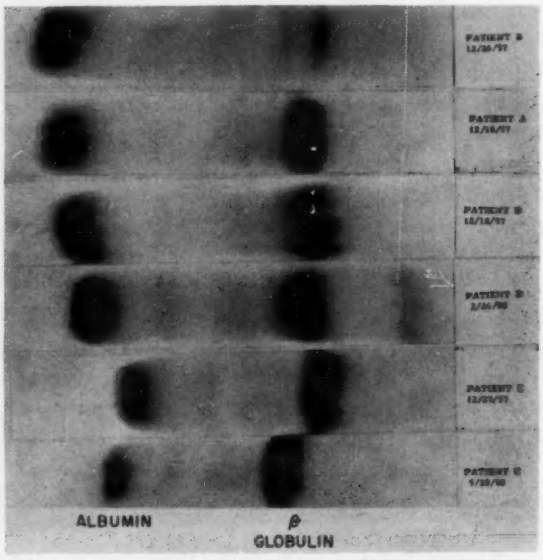



FIG. 1B. SERUM PAPER ELECTROPHORETIC PATTERN WITH BETA PEAK IN PATIENTS WITH MULTIPLE MYELOMA.

of 1 N HCl to the required number of millicuries (usually about 6) of $I^{131}$ obtained from Oak Ridge and an amount of iodine dissolved in KI (composition about $K_2I_4$) equivalent to 5 moles per mole of protein (assumed mol wt = 150,000). If appreciable decolorization of the iodine occurred, it was attributed to the sulfite preservative in the Oak Ridge iodine and subsequent yields were correspondingly low. The iodinating reagent was added slowly to the cold protein solution with continuous stirring and then allowed to stand for about 1 hour. Then it was passed slowly through two anion exchange columns (Abbott ioresin) of about 2 ml in volume to remove unbound iodine (iodide), the protein retained in the column being displaced by water and dialyzed overnight against cold isotonic sodium chloride. The preparations were diluted to a working volume and sterilized by passage through an asbestos filter of the Seitz type, and sterility proved by culture. Over 98 per cent of the radioactivity was precipitable by trichloroacetic acid. The yield was essentially quantitative in terms of the protein and from 5 to 8 per cent in terms of the radioactivity.

*Patients.* Ten patients with multiple myeloma were investigated. All diagnoses were established by microscopic examination of bone marrow aspirations. Chemical determinations of the serum proteins, paper electrophoretic serum patterns and X-ray bone surveys were also concordant with the diagnoses. The age of the patients ranged from 43 to 80 years and included both sexes and white as well as Negro individuals.

*Radioactive assay.* Counting was done in a well-type scintillation counter using a 2 inch NaI(Tl) crystal and standard electronic components. Duplicate 1 ml samples of serum were contained in ordinary 15 × 75 mm glass vials after simple separation in a centrifuge. Aliquots of the original solutions appropriately diluted were carried through the counting program for each experiment and all data are referred to these standards and reported as a percentage of the original dose. This of course automatically corrects for radioactive decay. By consideration of counting rates and background measurements, the counting error for the first part of the experiments was held to 1 per cent, while in the later points, the sample error approached 4 per cent at the lowest counting rates used.

*Radiation dosage.* In these experiments the intravenously administered radioactivity ranged from 7.5 to 22.1 μc. The maximal total dose was calculated to be 0.013 rads in a 50 kg patient, assuming an effective half-life of 4.3 days and taking the blood as the critical organ (13). Lugol's solution was started 3 days before injection of the labeled protein and given daily throughout the study to prevent thyroid accumulation of radioactive iodine if any were liberated from the compound. The attempt was made to use the smallest feasible quantities of radioactive label and to obtain maximal benefit of improvements in counting procedures and sampling techniques.

*Serum electrophoresis.* The paper electrophoresis was performed at pH 8.6 in a barbiturate buffer having an ionic strength of 0.05. The Whatman 1 MM paper strips were moistened with the buffer and placed in the electrophoretic apparatus which was then equilibrated for at least one-half hour. Ten λ(0.01 ml) of serum was placed on the Spinco applier with a micropipet and applied in a line transverse to the length of the filter paper. For 10 paper strips which were run in parallel, optimal separations were obtained with 150 to 170 v and 7 ma for 16 hours. The strips were dried in the oven at 110° C for 30 minutes and stained with 1 per cent bromphenol-blue in 95 per cent ethanol for 15 minutes. The strips were then washed 3 times in 1 per cent acetic acid baths. After drying at room temperature, the color intensity

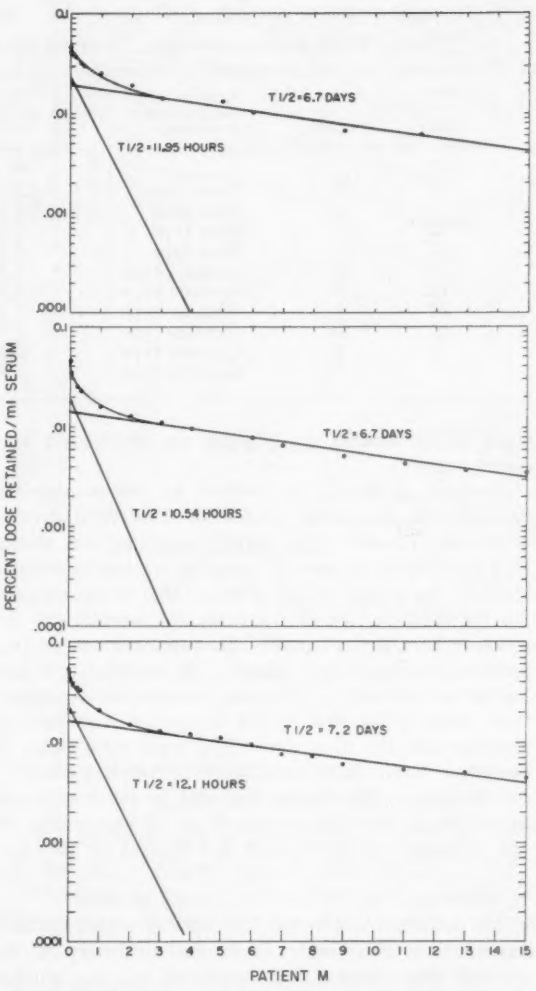



FIG. 2. SEMILOGARITHMIC PLOTS OF THREE DISAPPEARANCE CURVES OF $I^{131}$-LABELED NORMAL GAMMA GLOBULIN. From the sera of hypergammaglobulinemic Patient M with multiple myeloma in whom three turnover studies were made during a six month period.

TABLE I

*Single turnover of normal $I^{131}$-labeled gamma globulin in patients with multiple myeloma*

| Donor | Patient | Patient's serum electrophoretic pattern | Dose $I^{131}$ | Globulin injected | Duration of experiment | Start of experiment | $T_{\frac{1}{2}}$ slow component |
|---|---|---|---|---|---|---|---|
| | | | *μc* | *mg* | *days* | | *days* |
| I | D | Beta type | 22.1 | 6 | 18 | 11/1/57 | 14.6 |
| I | A | Beta type | 22.1 | 6 | 18 | 11/1/57 | 18.2 |
| II | B | Beta type | 7.5 | 7 | 12 | 11/20/57 | 17.5 |
| II | C | Beta type | 7.5 | 7 | 12 | 11/20/57 | 19.0 |
| II | K | Gamma type | 7.5 | 7 | 12 | 11/20/57 | 5.7 |
| II | M | Gamma type | 7.5 | 7 | 12 | 11/20/57 | 6.7 |
| II | J | Gamma type | 7.5 | 7 | 12 | 11/20/57 | 6.7 |
| II | G | Gamma type | 7.5 | 7 | 12 | 11/20/57 | 7.2 |
| II | H | Gamma type | 7.5 | 7 | 12 | 11/20/57 | 8.2 |
| II | N | Gamma type | 7.5 | 7 | 12 | 11/20/57 | 8.9 |

could be accentuated by exposing the strips over ammonia vapor.

*Analyses of data.* The method for determining the half-life from the serum concentration curve is demonstrated in Figure 2. The activity remaining was plotted as a function of the time of sampling on semilogarithmic paper. The plotted points were arrived at by comparing the counting rate of the particular sample with the counting rate of the initially administered tracer as represented by its diluted aliquot. To establish the biological half-life of the globulins, straight-line parameters were fitted to the data by the method of least squares, starting with the third day. The rapid early decay of the curve is considered to represent distribution and mixing processes. The decline indicated by the second part of the curve, the slow component, is an exponential decay indicated by the straight line derived in all cases. This exponential decay is believed to represent the rate of degradation of the injected gamma globulin.

The activity found in the daily urinary output has been expressed in two ways: *1*) as the percentage of the original dose excreted daily, corrected only for physical decay, and *2*) as the ratio of the percentage excreted daily to that remaining in the body on that day. The first method of calculation is referred to as the *excretion rate* and the second as the *degradation rate*. From the first procedure the cumulative urinary excretion for any period of days may be determined. No determinations were made of radioactivity appearing in the stool although it has been reported (14) that for $I^{131}$-labeled albumin, fecal excretion can account for as much as 2.5 per cent of that excreted in the urine and, in addition, that up to 3 per cent of the radioactivity may be withdrawn during blood sampling in the course of a study. Thus the assumption is that about 95 per cent or more of all the radioactivity lost from the body is recovered in the urine. Incompleteness of urinary collections, as in bed patients voiding into a bed pan, must be taken into consideration in evaluating excretion data. An additional computation described (14) as a "distribution curve" was determined by dividing the serum concentration curve by the percentage of activity retained in the body. According to these authors the significance of a horizontal asymptote in the distribution curve is twofold: *1*) it offers evidence that during the entire period of study, radioiodine released from degraded protein is almost completely recovered in the urine; and *2*) it further indicates that any change in the slope of the plasma concentration curve after the distribution curve has become horizontal must be due to a change in the apparent rate of degradation and is not to be explained by distribution or storage phenomena. Degradation rates determined from two independent sets of data, plasma concentrations, and urinary excretions may thus be compared.

## RESULTS

The serum electrophoretic patterns of the patients with multiple myeloma are shown in Figures 1 A and B. In four of these (B) the predominant peak was of the beta type and in the

TABLE II

*Repeated turnovers of normal $I^{131}$-labeled gamma globulin in patients with multiple myeloma*

| Donor | II | II | II | II | II | II | II | II | II | II |
|---|---|---|---|---|---|---|---|---|---|---|
| Patient | C | C | C | M | M | M | G | G | N | N |
| Electrophoretic pattern | Beta | Beta | Beta | Gamma | Gamma | Gamma | Gamma | Gamma | Gamma | Gamma |
| Dose $I^{131}$, μc | 7.5 | 20.0 | 21.8 | 7.5 | 20.0 | 21.8 | 7.5 | 21.8 | 7.5 | 14.7 |
| Mg γ-globulin injected | 7 | 10 | 5.3 | 7 | 10 | 5.3 | 7 | 5.3 | 7 | 3.5 |
| Duration experiment, days | 12 | 17 | 16 | 12 | 17 | 16 | 12 | 16 | 12 | 17 |
| Date start of experiment | 11/20/57 | 5/1/58 | 5/29/58 | 11/29/57 | 5/1/58 | 5/29/58 | 11/20/57 | 5/29/58 | 11/20/57 | 5/28/58 |
| $T_{\frac{1}{2}}$ slow component, days | 19.0 | 11.4* | 16.1 | 6.7 | 6.7 | 7.2 | 7.2 | 6.9 | 8.9 | 7.2 |

* On urethan therapy at this time.

remaining six (A) of the gamma type. The half-life of each of the slow components is presented in Table I. Two normal donor sources of gamma globulin were utilized and the amount of protein administered was so small that it could not have had any significant effect on the total protein concentration or metabolism in the patient. The amount of $I^{131}$ used for labeling was sufficient for counting purposes but minimal in amount so that there would be insufficient radiation to degrade the labeled protein and so that the whole-body radiation dose would be considerably below the allowed level of rads for a tracer investigation. The mean of the half-life of the slow component obtained from the serum concentration curves for the beta type was $16.12 \pm 1.15$ days and for the gamma type $7.14 \pm 0.28$ days.[1] The probability was less than 0.001 when these latter two groups were compared.

Since it is impossible to do replicate testing in determining the accuracy of the method of obtaining the half-life, repeated turnovers were done as is illustrated for one case in Figure 2 and for four cases in Table II. This method offered the additional advantage of further testing the homogeneity of the globulin preparations and the reproducibility of the iodination technique. In Patients M, G and N the comparative data are quite consistent for the half-life of the slow components. In Patient C in the slow component the half-life for the second turnover was 11.4 days in contrast to 19 and 16.1 days, respectively, for the first and third turnovers. No explanation for this has been established, but it is of interest that at that time the patient was on urethan therapy.

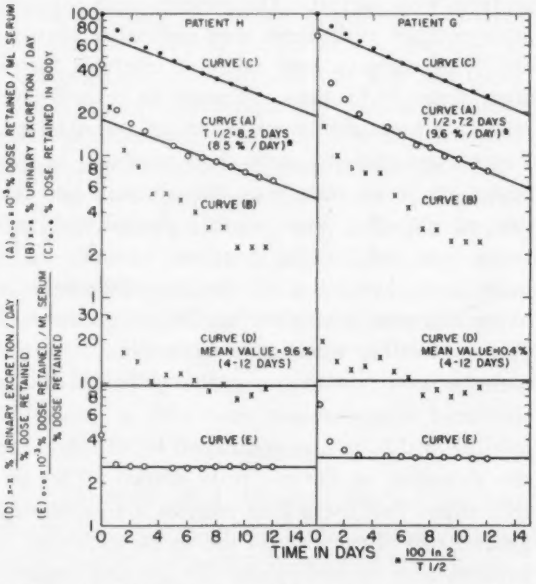



FIG. 3. TURNOVER OF NORMAL GAMMA GLOBULIN IN TWO HYPERGAMMAGLOBULINEMIC MULTIPLE MYELOMA PATIENTS.

[1] Obtained from Tables I and II.

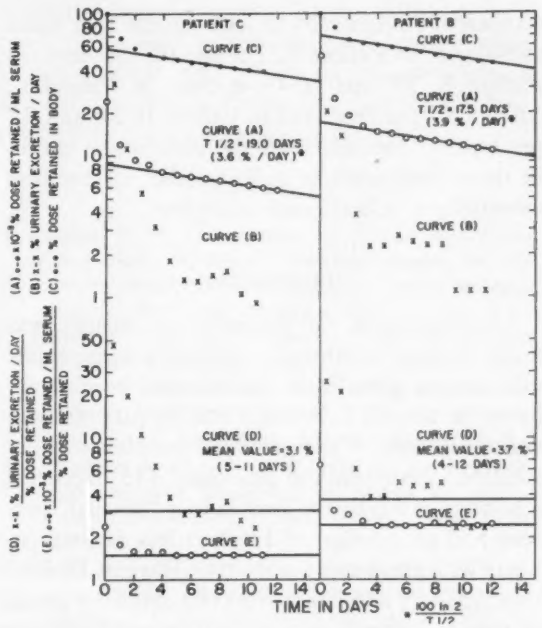



FIG. 4. TURNOVER OF NORMAL GAMMA GLOBULIN IN TWO HYPERBETAGLOBULINEMIC MULTIPLE MYELOMA PATIENTS.

It has been stated earlier in this paper that the homogeneity of the gamma globulins obtained from fractionation of serum from healthy individuals and utilized in these studies was investigated by determining certain of their physicochemical characteristics. Limited further information on the homogeneity or lack of it in the $I^{131}$-labeled gamma globulin used for the turnover studies was obtained by determination of the urinary radioiodine excretion in four patients. Two patients had a hypergammaglobulinemia serum pattern (Figure 3) and the other two a hyperbetaglobulinemia pattern (Figure 4). The degradation rates determined from the serum concentration curves and the urinary excretions may be

compared, respectively, in each of the four cases as follows: in Patient H, 8.5 and 9.6 per cent; in Patient G, 9.6 and 10.4 per cent; in Patient C, 3.6 and 3.1 per cent; and in Patient B, 3.9 and 3.7 per cent. Examination of the distribution curves in these four subjects indicates that all conform essentially to a horizontal asymptote.

## DISCUSSION

In a survey of the literature an attempt was made to learn whether, in properly prepared normal gamma globulin, a half-life had been established in normal individuals and in patients with various diseases. Very little information was obtainable. Vaughan and associates (15) reported a half-life of 7.0 to 8.8 days in four "normal" persons and an average of 1.8 days less in four patients with rheumatoid arthritis. Havens, Dickensheets, Bierly and Eberhard (16) studied a group of nine individuals consisting of normal subjects and convalescent patients in whom the half-life was 10.6 to 16.6 days. Eisenmenger and Slater (17) in eight "normals" found a half-life of 11.5 to 19 days and in 25 patients with cirrhosis, 6 to 13 days. In two of these three studies, the half-life for normal individuals ranged from 10.6 to 19 days, somewhat comparable (slightly shorter) to our beta type multiple myeloma patients. In one study the turnover was short, 7.0 to 8.8, and raises the question of whether or not the labeled protein had undergone degradation. In the two disease states studied, the half-life was markedly shortened as it was in our gamma type multiple myeloma patients.

Serum protein turnover in two patients with multiple myeloma has been studied by Berson and Yalow (18). The serum proteins of a patient with a high serum concentration of gamma myeloma globulin but no Bence Jones proteinuria were labeled with $I^{131}$ and administered intravenously to this subject as well as to another patient who manifested marked Bence Jones proteinuria but was not considered to demonstrate anomalous serum proteins in his electrophoretic pattern. The half-time of the donor's myeloma protein given to himself was 15.5 days, and in the recipient with the presumably normal serum protein distribution it was 17 days. This experiment differs from ours in that we fractionated normal gamma globulin from the sera of healthy control individuals, labeled it with the same isotope, $I^{131}$, and determined the half-life of this material in patients with multiple myeloma. Thus we cannot make a comparative statement, but it is of interest that in our patients with a gamma peak demonstrated electrophoretically in the serum, the range of half-life was 5.7 to 8.9 days for normal gamma globulin, while in Berson and Yalow's gamma type myeloma patient, the turnover for the labeled serum myeloma protein was much longer—15.5 days. In our unpublished data on six patients with electrophoretic gamma type serum peaks, injected with $I^{131}$-labeled homologous gamma globulin preparations from patients with multiple myeloma, the range of half-life was 5.5 to 9.2 days. In both of our types of preparations the half-life in patients with a gamma type serum peak was significantly shorter than that in Berson and Yalow's two cases.

Neufeld (19) carried out a study on two patients with multiple myeloma, one with a gamma and the other with a beta type serum electrophoretic pattern. One hundred $\mu$c of $I^{131}$ was added to plasma obtained from each patient so that, according to the author, the patients' own serum proteins were tagged. The patients were injected intravenously with their own iodinated plasma, and blood samples were taken at intervals thereafter through 15 days. Counts in a well-type scintillation counter were made on the samples. The plasma proteins were then separated by repeated electrophoretic runs, the albumin and the lumped globulins were eluted, precipitated and taken into solution to constant volume. The count was taken for each. Semilogarithmic plots of the corrected counts per minute per milliliter of plasma globulins were made against time. In the patients with myelomatosis, the globulins had a shortened disappearance rate with a mean $I^{131}$ half-life of 7.2 days, as contrasted to 10.6 days for the globulins of normal male adults. The author states that these data suggest a significantly greater rate of turnover of the serum globulins in patients with myelomatosis. In the first patient, 6.7 per cent of his total serum globulin was represented by abnormal gamma globulin, while in the second patient, 72 per cent was in the beta globulin. In our unpublished data on $I^{131}$-labeled

gamma globulin, obtained from patients with multiple myeloma, whose half-life was determined autologously and homologously as referred to above, the half-life is in agreement in the gamma type patient with Neufeld's findings.

In evaluating the significance of the difference in half-life of labeled normal gamma globulin in our multiple myeloma patients with beta and gamma type serum electrophoretic patterns, several factors have to be considered. The term "normal gamma globulin" refers to two preparations fractionated from two healthy donors. Even under the best methods of fractionation it is recognized that a small percentage of heterogeneity will be present in the final product used for labeling. The purpose in using physicochemical techniques for studying these preparations is to select what appear to be the best of them. That $I^{131}$, even in the small amounts used for labeling, may conceivably alter the normal preparations is recognized. Radiation itself from the isotope may affect the protein but in the amounts used in these studies this effect must, at most, be very little. The excretion rates determined in four patients receiving the same labeled globulin indicate a variation in rate which, in the first 24 hours varied from 22.70 per cent in one gamma type individual to 31.66 per cent in a beta type patient. This may be a further indication of some degree of heterogeneity. Finally, 18 was the maximum number of days for which any patient was followed to determine a half-life. If ten times as much tracer $I^{131}$ had been used in our investigations, the slow component could have been determined in studies extending over a longer period of time. Under such a situation it is conceivable that the half-life in both the beta and gamma type patients might be longer, but it is doubtful that this would alter the significant differences in half-life in both groups.

## SUMMARY

The turnover of $I^{131}$-labeled normal gamma globulin was determined in ten patients with multiple myeloma and correlated with the serum globulin distribution as established by paper electrophoresis. In the beta type individuals the mean of the half-life was $16.12 \pm 1.15$ days, and for the gamma type, $7.14 \pm 0.28$ days. The rate of degradation for normal gamma globulin indicated by the slow component was far more rapid for the patient with excessive gamma globulin in his serum protein distribution than for the individual with excessive beta globulin.

## REFERENCES

1. Putnam, F. W. Aberrations of protein metabolism in multple myeloma. Interrelationships of abnormal serum globulins and Bence-Jones proteins. Physiol. Rev. 1957, **37**, 512.
2. Columbia University College of Physicians and Surgeons Combined Staff Clinic. Multiple myeloma. Current clinical and chemical concepts. Amer. J. Med. 1957, **23**, 283.
3. Smith, E. L., Brown, D. M., McFadden, M. L., Buettner-Janusch, V., and Jager, B. V. Physical, chemical, and immunological studies on globulins from multiple myeloma. J. biol. Chem. 1955, **216**, 601.
4. Slater, R. J., Ward, S. M., and Kunkel, H. G. Immunological relationships among the myeloma proteins. J. exp. Med. 1955, **101**, 85.
5. Putnam, F. W. N-terminal groups of normal human gamma globulin and of myeloma proteins. J. Amer. chem. Soc. 1953, **75**, 2785.
6. Grisolia, F. T., and Cohen, P. P. Amino acid analysis of serum proteins in multiple myeloma. Cancer Res. 1953, **13**, 851.
7. Adams, W. S., Alling, E. L., and Lawrence, J. S. Multiple myeloma. Its clinical and laboratory diagnosis with emphasis on electrophoretic abnormalities. Amer. J. Med. 1949, **6**, 141.
8. Conn, H. O., and Klatskin, G. Filter paper electrophoretic patterns of serum in multiple myeloma. Amer. J. Med. 1954, **16**, 822.
9. Eriksen, N., Ellerbrook, L. D., and Lippincott, S. W. Serum protein analysis by electrophoresis and by the Wolfson-Cohn chemical method. Comparative study. Clin. Chem. 1956, **2**, 334.
10. Reiner, M., and Stern, K. G. Electrophoretic studies on the protein distribution in the serum of multiple myeloma patients. Acta Haemat. (Basel) 1953, **9**, 19.
11. Nichol, J. C., and Deutsch, H. F. Biophysical studies of blood plasma proteins. VII. Separation of gamma-globulin from the sera of various animals. J. Amer. chem. Soc. 1948, **70**, 80.
12. Hughes, W. L., Jr., and Straessle, R. Preparation and properties of serum and plasma proteins. XXIV. Iodination of human serum albumin. J. Amer. chem. Soc. 1950, **72**, 452.
13. Hine, G. J., and Brownell, G. L., Eds. Radiation Dosimetry. New York, Academic Press Inc. 1956, p. 223.
14. Berson, S. A., Yalow, R. S., Schreiber, S. S., and Post, J. Tracer experiments with $I^{131}$ labeled hu-

man serum albumin: Distribution and degradation studies. J. clin. Invest. 1953, **32**, 746.

15. Vaughan, J. H., Armato, A., Goldthwait, J. C., Brachman, P., Favour, C. B., and Bayles, T. B. A study of gamma globulin in rheumatoid arthritis. J. clin. Invest. 1955, **34**, 75.
16. Havens, W. P., Dickensheets, J., Bierly, J. N., and Eberhard, T. P. The half-life of normal human gamma globulin in patients with hepatic cirrhosis (abstract). J. clin. Invest. 1953, **32**, 573.
17. Eisenmenger, W. J., and Slater, R. J. Distribution and decay of $I^{131}$ tagged albumin and gamma globulin in patients with cirrhosis (abstract). J. clin. Invest. 1953, **32**, 564.
18. Berson, S. A., and Yalow, R. S. Serum protein turnover in multiple myeloma. J. Lab. clin. Med. 1957, **49**, 386.
19. Neufeld, A. H. A new concept of the metabolism of the pathological proteins in myelomatosis. Canad. J. Biochem. 1957, **35**, 977.

## ANNOUNCEMENT OF MEETINGS

The Fifty-Second Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Sunday afternoon and evening, May 1, 1960, in the Chalfonte-Haddon Hall (jointly with the AFCR); and on Monday, May 2, at 9:00 A.M., at the Casino Theater on the Steel Pier.

The Seventeenth Annual Meeting of THE AMERICAN FEDERATION FOR CLINICAL RESEARCH will be held in Atlantic City, N. J., on Sunday, May 1, 1960, at 9:00 A.M., at the Casino Theater on the Steel Pier. On Sunday afternoon and evening, May 1, 1960, a joint sectional meeting with The American Society for Clinical Investigation will be held in rooms in the Chalfonte-Haddon Hall.

THE ASSOCIATION OF AMERICAN PHYSICIANS will hold its Seventy-Third Annual Meeting in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Tuesday, May 3, 1960, at 9:30 A.M., and in the Vernon Room, Chalfonte-Haddon Hall, on Wednesday, May 4, 1960, at 9:30 A.M.

# SOME EFFECTS OF RESTRICTION OF CHEST CAGE EXPANSION ON PULMONARY FUNCTION IN MAN: AN EXPERIMENTAL STUDY*†

By COLIN G. CARO, JOHN BUTLER‡ AND ARTHUR B. DuBOIS§

*(From the Department of Physiology of the Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.)*

(Submitted for publication April 15, 1959; accepted December 4, 1959)

Certain alterations in pulmonary mechanics occur in man when an inspiration is taken from below the resting lung volume (1) and during shallow breathing (2). Some effects of a sustained reduction of the total lung capacity on respiration have been studied previously (3–5), yet the effects of chest strapping on pulmonary mechanics have received relatively little attention.

In the preliminary experiments we tightly bound the chest in the expiratory position with either adhesive tape or a long strip of rubber. Subsequently we designed a special chest corset that permitted us to reduce the resting lung volume and inspiratory capacity at will. The entire pressure-volume curve of the lung was altered by these procedures, but was restored to normal when a deep inspiration was taken after release of the restricting apparatus. Simultaneously, the escape of gas from poorly ventilated regions of the lung was detected. The effect of these changes on other aspects of pulmonary and circulatory function was examined and our findings, together with a consideration of their physiological and clinical significance, form the basis of this report.

## METHOD

Twenty-five normal subjects were studied, all but two of them males. They ranged in age from 19 to 40 years. The thoracic cage was tightly strapped in the position of full expiration with one of the following: *1*) two inch inextensible adhesive tape (Figure 1A); *2*) a long strip, one inch wide, of rubber inner tubing wound several times about the chest, referred to as the extensible method (Figure 1B); or *3*) an adjustable chest corset (made by Henry Saur Co., Inc., Philadelphia, Pa.; Figure 2). In a few subjects the abdomen alone was restricted with the rubber strip. All measurements were made with the subject in the seated position.

The vital capacity (VC) and its subdivisions were measured with a recording spirometer, while the functional residual capacity (FRC) was determined by means of a body plethysmograph (6). A Tissot spirometer and kymograph was used to record the minute volume of ventilation, respiratory frequency and tidal volume. Volumes were corrected to BTPS.

A nitrogen gas analyzer was used to detect uneven alveolar ventilation following a single breath of oxygen (7). To measure the peripheral venous pressure, a glass tube manometer filled with 3.8 per cent sodium citrate solution was attached to an 18 gauge needle inserted into a subject's median antecubital vein. The initial reading made with reference to the manubrial angle was compared with a measurement, again made sitting, after the chest was strapped.

The pressure-volume relationship of the lung was studied with an air-containing esophageal balloon (8), attached via 1 mm ID polyethylene tubing to a capacitance manometer. The esophageal pressure was registered on a direct-writing Brush recorder and on a cathode ray oscillograph.

Several methods were used to measure the lung volume. In studying the *static* pressure-volume relationship of the lung, the thoracic gas volume was determined

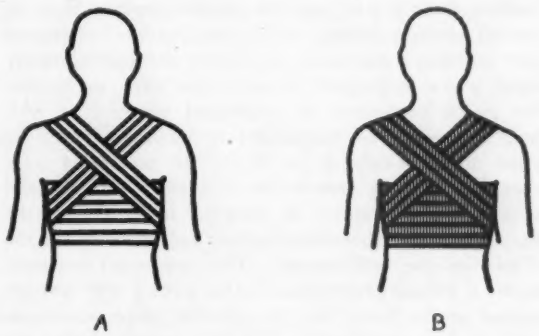



FIG. 1. METHODS OF CHEST RESTRICTION: A, ADHESIVE TAPE; B, STRIP OF RUBBER INNER TUBING.

* Aided by a grant from The National Foundation.

† Presented in preliminary form at the Autumn Meeting of the American Physiological Society, 1958.

‡ This work was done during the tenure of a Rockefeller Traveling Fellowship in Medicine. Present address: Department of Medicine, University of Manchester, Manchester, England.

§ Established Investigator of the American Heart Association.

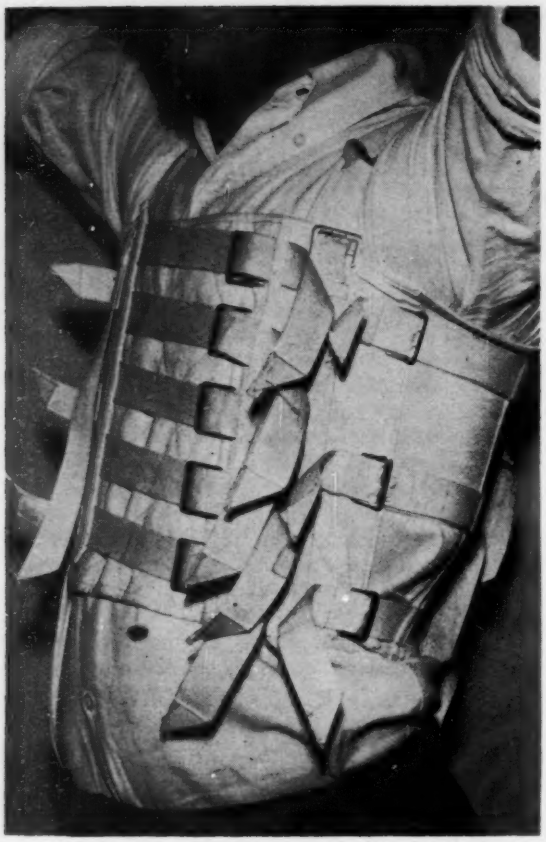


FIG. 2. PHOTOGRAPH OF A SUBJECT WEARING CHEST CORSET. The corset was made of coutil with 26 gauge rubber side panels. One inch web straps with prong buckles were used to join the two sections back and front, as shoulder straps, and to bridge over the rubber side panels.

by means of the body plethysmograph (6) at different respiratory levels over the range of the vital capacity. The corresponding mean esophageal pressure during shallow panting was recorded simultaneously. Since in normal subjects during shallow panting the inspiratory and expiratory nonelastic resistances are approximately equal, mean esophageal pressure was taken to indicate the elastic component of esophageal pressure at each lung volume. The fluctuations in esophageal pressure were approximately 2 cm $H_2O$. In measuring lung compliance during *spontaneous* breathing (8) the tidal volume was determined by electrical integration of the signal from the pneumotachygraph and recorded on the Y-axis of the oscillograph. The esophageal pressure, minus a voltage proportional to the airflow (9) was recorded on the X-axis and the resulting slope was measured as lung compliance. The tidal volume during *nitrogen dilution procedures* was measured from the pressure changes registered by a capacitance manometer attached to a box containing a breathing bag (i.e. a Donald-Christie box). This system had no detectable phase lag at the breathing frequencies encountered, and lung compliance was calculated from the tidal volume and esophageal pressure tracings. A recording spirometer was connected to the Donald-Christie box via a stopcock and used to measure the volume of deep breaths, thus avoiding large pressure changes within the system. In studying the *time course* of the onset of and recovery from lung compliance changes, the tidal volume and changes in resting lung volume were recorded with a closed circuit spirometer system. Esophageal pressure was measured at points of no airflow indicated by the pneumotachygraph recording.

The airway conductance is defined as the rate of airflow at the mouth per unit of alveolar-mouth pressure gradient. Airway conductance was measured during shallow panting by means of a body plethysmograph and compared with thoracic gas volume (10, 11). Observations were made at the resting lung volume and at other lung volumes over the range of the vital capacity.

To test for the presence of underventilated gas within the lungs during chest restriction, nitrogen dilution curves were plotted and analyzed for the release of nitrogen after removal of the restriction about the chest (12) and after a deep inspiration of oxygen. Additional details of these procedures are given with the results.

The oxygen tension of samples of brachial arterial blood was measured in control experiments in subjects who had breathed medical oxygen from a nonreturn valve system for periods of from 15 to 60 minutes. While the subjects continued to breathe oxygen, the chest cage was strapped and samples were drawn at intervals for as long as 45 minutes after the onset of chest restriction and after the restriction was released. The partial pressure of oxygen in the arterial samples was measured by means of a modification (13) of the Clark electrode. Measurement of arterial oxygen tension, for further investigating the previously reported effects of chest cage restriction on pulmonary mechanics (14), was suggested by other workers (15).

## RESULTS

*Lung volumes.* The changes in the subdivisions of the total lung capacity produced by chest strapping are recorded in Table I. The average residual volume was not significantly changed; however, both the inextensible and extensible methods of chest strapping reduced the functional residual capacity and hence reduced the expiratory reserve volume. The inspiratory capacity was most markedly decreased by the inextensible strapping and it follows that the greatest reduction of the vital capacity and total lung capacity occurred with this latter method. Similar but less marked changes followed abdominal strapping (Table I).

TABLE I

*Effect of chest restriction on subdivisions of total lung capacity* *

| Subject | Method of restriction: Chest restricted | Total lung capacity | | Subdivisions of total lung capacity: Vital capacity | | Inspiratory capacity | | Expiratory reserve volume | | Functional residual capacity | | Residual volume | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Control | Restricted | Control | Restricted | Control | Restricted | Control | Restricted | Control | Restricted | Control | Restricted |
| | | *L* | *L* | *L* | *L* | *L* | *L* | *L* | *L* | *L* | *L* | *L* | *L* |
| AD† | Tape | 8.20 | 2.80 | 5.50 | 0.80 | 3.65 | 0.20 | 1.85 | 0.60 | 4.55 | 2.60 | 2.70 | 2.00 |
| CC | Tape | 6.33 | 3.18 | 5.18 | 1.70 | 2.96 | 1.03 | 2.44 | 0.51 | 3.37 | 2.15 | 0.93 | 1.64 |
| SS | Tape | 6.69 | 3.28 | 6.00 | 1.62 | 2.91 | 0.66 | 2.50 | 0.96 | 3.78 | 2.62 | 1.28 | 1.62 |
| NS | Tape left hemithorax | 6.75 | 4.28 | 5.15 | 2.85 | 3.50 | 1.70 | 1.60 | 1.15 | 3.25 | 2.58 | 1.65 | 1.43 |
| Mean | | 6.99 | 3.39 | 5.46 | 1.74 | 3.26 | 0.90 | 2.10 | 0.81 | 3.74 | 2.49 | 1.64 | 1.67 |
| SE | | 0.41 | 0.32 | 0.20 | 0.42 | 0.19 | 0.32 | 0.22 | 0.15 | 0.29 | 0.11 | 0.38 | 0.12 |
| PB | Rubber | 6.57 | 5.52 | 5.37 | 4.43 | 3.56 | 3.57 | 1.83 | 0.87 | 3.01 | 1.95 | 1.18 | 1.08 |
| GL | Rubber | 6.12 | 3.77 | 4.40 | 2.04 | 2.70 | 1.42 | 1.69 | 0.58 | 3.42 | 2.35 | 1.73 | 1.77 |
| GP | Rubber | 4.91 | 4.19 | 4.03 | 3.08 | 2.71 | 2.67 | 1.26 | 0.39 | 2.20 | 1.52 | 0.94 | 1.13 |
| DM | Rubber | 6.13 | 4.55 | 4.68 | 3.31 | 3.61 | 2.81 | 1.02 | 0.50 | 2.52 | 1.74 | 1.50 | 1.24 |
| JB | Rubber | 8.08 | 6.33 | 5.70 | 4.80 | 4.18 | 4.23 | 1.52 | 0.57 | 3.90 | 2.10 | 2.38 | 1.53 |
| TS | Corset | 6.60 | 5.90 | 4.90 | 4.10 | 3.05 | 3.15 | 1.85 | 0.95 | 3.55 | 2.75 | 1.70 | 1.80 |
| ST† | Rubber | 4.20 | 2.60 | 3.00 | 1.45 | 1.86 | 1.04 | 1.14 | 0.41 | 2.34 | 1.56 | 1.20 | 1.15 |
| CC† | Rubber | 6.90 | 4.20 | 4.90 | 2.70 | 3.36 | 1.92 | 1.54 | 0.78 | 3.54 | 2.28 | 2.00 | 1.50 |
| HL† | Rubber (very tight) | 7.70 | 1.90 | 5.60 | 0.65 | 3.75 | 0.00 | 1.85 | 0.82 | 3.95 | 2.07 | 2.10 | 1.25 |
| AD† | Rubber | 7.50 | 6.60 | 5.40 | 4.70 | 3.28 | 4.02 | 2.12 | 0.68 | 4.22 | 2.58 | 2.10 | 1.90 |
| Mean | | 6.47 | 4.56 | 4.80 | 3.13 | 3.21 | 2.48 | 1.58 | 0.66 | 3.27 | 2.09 | 1.68 | 1.44 |
| SE | | 0.38 | 0.49 | 0.26 | 0.45 | 0.21 | 0.43 | 0.11 | 0.06 | 0.23 | 0.13 | 0.15 | 0.10 |
| Mean, all subjects (chest restricted) | | 6.62 | 4.22 | 4.99 | 2.73 | 3.22 | 2.03 | 1.73 | 0.70 | 3.40 | 2.20 | 1.67 | 1.50 |
| SE | | 0.28 | 0.38 | 0.21 | 0.38 | 0.15 | 0.37 | 0.17 | 0.06 | 0.19 | 0.11 | 0.15 | 0.08 |
| | Abdomen restricted | | | | | | | | | | | | |
| CC† | Rubber | 7.20 | 5.50 | 5.00 | 3.30 | 3.47 | 2.53 | 1.53 | 0.77 | 3.73 | 2.97 | 2.20 | 2.20 |
| JB† | Rubber | 5.50 | 4.20 | 3.50 | 2.80 | 2.37 | 2.40 | 1.13 | 0.40 | 3.13 | 1.80 | 2.00 | 1.40 |
| KA† | Rubber | 6.20 | 5.80 | 4.90 | 4.40 | 3.23 | 3.59 | 1.67 | 0.81 | 2.97 | 2.21 | 1.30 | 1.40 |
| Mean | | 6.30 | 5.17 | 4.47 | 3.50 | 3.02 | 2.84 | 1.44 | 0.66 | 3.28 | 2.33 | 1.83 | 1.67 |
| SE | | 0.49 | 0.49 | 0.49 | 0.47 | 0.33 | 0.38 | 0.16 | 0.13 | 0.23 | 0.35 | 0.27 | 0.27 |

* Pressure-volume relationships of the lung, obtained during these experiments, are listed in Table III.
† Subdivisions of total lung capacity were measured plethysmographically.

TABLE II

*Effect of chest restriction on minute volume, respiratory frequency and tidal volume*

| Subject | Method of restriction | Minute volume | | Respiratory frequency | | Tidal volume | |
|---|---|---|---|---|---|---|---|
| | | Control | Restricted | Control | Restricted | Control | Restricted |
| | | *L/min* | | *rpm* | | *L* | |
| SSa* | Rubber | 7.9 | 8.8 | 12 | 17 | 0.66 | 0.52 |
| JB* | Rubber | 12.0 | 13.3 | 19 | 20 | 0.63 | 0.67 |
| ER† | Corset | 10.8 | 10.6 | 14 | 22 | 0.77 | 0.48 |
| RS† | Corset | 10.3 | 9.5 | 27 | 32 | 0.38 | 0.30 |
| JJ† | Corset | 10.6 | 10.1 | 11 | 17 | 0.96 | 0.59 |
| EL† | Corset | 12.4 | 11.3 | 25 | 29 | 0.50 | 0.39 |
| GJ† | Corset | 8.8 | 9.7 | 18 | 23 | 0.49 | 0.42 |
| LM† | Corset | 12.1 | 14.0 | 20 | 29 | 0.60 | 0.48 |
| LU† | Corset | 9.6 | 8.8 | 18 | 21 | 0.53 | 0.42 |
| Mean | | 10.5 | 10.7 | 18 | 23 | 0.61 | 0.47 |
| SE | | 0.51 | 0.62 | 1.8 | 1.8 | 0.06 | 0.04 |

* Breathing air.
† Breathing oxygen during measurement of arterial oxygen tension.

Chest strapping caused an increase of respiratory frequency and a decrease of tidal volume. The minute volume of ventilation was, however, not significantly altered (Table II).

*Pressure-volume relationship of the lung.* The data obtained on a single subject (PB, Table III) have been plotted in Figures 3A and 3B to illustrate the effect of chest strapping on the static

TABLE III

*Effect of chest restriction on pressure-volume relationship of the lung* *

| Subject | Method of restriction Chest restricted | Lung compliance† | | | Trans-pulmonary pressure‡ at lung volume = control FRC | | | Maximal trans-pulmonary pressure‡ | |
|---|---|---|---|---|---|---|---|---|---|
| | | Control | Restricted: At control FRC | Restricted: At reduced FRC | Control | Restricted | After release of restriction, but prior to full inspiration | Control | Restricted |
| | | *L/cm H₂O* | | | *cm H₂O* | | | *cm H₂O* | |
| AD | Tape | 0.36 | | 0.11 | 5.1 | | 6.2 | 13 | 7 |
| CC | Tape | 0.22§ | | 0.08‡ | | | | | |
| SS | Tape | 0.25 | | 0.10 | 11.9 | | | 19 | 11 |
| NS | Tape Left Hemithorax | 0.19§ | | 0.17‡ | | | | | |
| Mean | | 0.25 | | 0.11 | 8.5 | | 6.2 | 16 | 9 |
| SE | | 0.04 | | 0.02 | | | | | |
| PB | Rubber | 0.33 | 0.19 | 0.15 | 0.0 | 6.7 | 12.3 | 20 | 17 |
| GL | Rubber | 0.34 | | 0.22 | 4.2 | | | 10 | 7 |
| GP | Rubber | 0.16 | 0.10 | 0.13 | 4.0 | 9.2 | 4.6 | 25 | 17 |
| DM | Rubber | 0.23 | 0.18 | 0.18 | 6.4 | 7.3 | 10.4 | 27 | 11 |
| JB | Rubber | 0.34 | 0.30 | 0.24 | 2.4 | 9.2 | 4.8 | 20 | 12 |
| TS | Corset | 0.28 | 0.43 | 0.19 | 1.6 | 7.2 | | 11 | 11 |
| ST | Rubber | 0.12 | 0.04 | 0.09 | 7.3 | 15.0 | | 26 | 20 |
| CC | Rubber | 0.22 | 0.18 | 0.10 | 3.9 | 10.2 | | 25 | 15 |
| HL | Rubber (very tight) | 0.28 | | 0.08 | 4.1 | | 7.2 | 20 | 8 |
| AD | Rubber | 0.48 | 0.48 | 0.29 | 6.3 | 7.5 | | 26 | 16 |
| Mean | | 0.28 | 0.24 | 0.17 | 4.0 | 9.0 | 7.9 | 21 | 13 |
| SE | | 0.03 | 0.05 | 0.02 | 0.72 | 0.96 | 1.53 | 1.6 | 1.3 |
| Mean, all subjects (chest restricted) | | 0.27 | 0.24 | 0.15 | 4.8 | 9.0 | 7.6 | 20 | 13 |
| SE | | 0.025 | 0.054 | 0.055 | 0.88 | 0.96 | 1.28 | 1.7 | 1.2 |
| | Abdomen restricted | | | | | | | | |
| CC | Rubber | 0.22 | 0.28 | 0.23 | 1.7 | 3.3 | | 17 | 10 |
| JB | Rubber | 0.23 | 0.29 | 0.09 | 4.1 | 12.8 | | 23 | 16 |
| KA | Rubber | 0.37 | 0.82 | 0.18 | 4.6 | 4.6 | | 23 | 14 |
| Mean | | 0.30 | 0.46 | 0.17 | 3.5 | 6.9 | | 21 | 13 |
| SE | | 0.03 | 0.18 | 0.04 | 0.9 | 3.0 | | 1.9 | 1.9 |

* For subdivisions of total lung capacity corresponding experiments see Table I.
† Measured in range to tidal volume from P-V curves.
‡ Transpulmonary pressure = mouth pressure (atmospheric pressure) minus esophageal pressure.
§ Lung compliance measured during spontaneous breathing.

pressure-volume relationship of the lung. An S-shaped line (——) has been drawn by inspection through points (●) obtained in random order over the range of the vital capacity during the control period. After the chest was strapped new points (○) were obtained, again in random order, over the diminished ranges of the vital capacity and of transpulmonary pressure (mouth pressure minus esophageal pressure) and a curve (- - - -) has been drawn through them. The latter points were displaced downward and to the right of the former. When the strapping was released the subject permitted his resting lung volume to return to normal but breathed quietly and without taking in a deep breath. New points (×) were then obtained at progressively increasing values of lung volume until the fullest possible inspiratory volume was reached and further points (×) were thereafter obtained at values of progressively decreasing lung volume. The direction of the curves (·——·) drawn through these points is indicated by the arrows on the diagram. They form a loop and the descending curve approaches and approximates the control curve.

The essential features were a shift in the pressure-volume curve of the lung when the chest was strapped and failure of the pressure-volume relationship of the lung to return to normal until a high inspiratory volume was reached after release of the strapping. Similar data were obtained on several additional subjects whose chests or abdomens were strapped (Table III). Because of the sigmoid shape of the pressure-volume curve, the lung compliance slope varied after strapping depending upon whether it was measured on a steep or a flat part of the S (Table III). However, transpulmonary pressure measured at a lung volume corresponding to control functional residual capacity was significantly increased. The mean transpulmonary pressure in eight subjects was 4.0 cm $H_2O$, SE 0.72, before chest strapping and 9.0 cm $H_2O$, SE 0.96, while strapped ($p = 0.006$). Both measurements were made at a lung volume corresponding to control FRC (Table III). This indicated that there had been a significant separation of the curves.

It was also shown in several subjects that the transpulmonary pressure, measured at a lung volume corresponding to control FRC, remained increased after the chest strapping was released. Thus the mean transpulmonary pressure in six subjects at a lung volume corresponding to the control FRC was 3.7 cm $H_2O$, SE 0.91, prior to chest strapping and 7.6 cm $H_2O$, SE 1.28 ($p = 0.006$), at the same volume after the restriction was released (Table III). The lung compliance was measured during spontaneous breathing and during breathing at 40 to 50 respirations per minute in four subjects in the control period and then when their chests were strapped; the

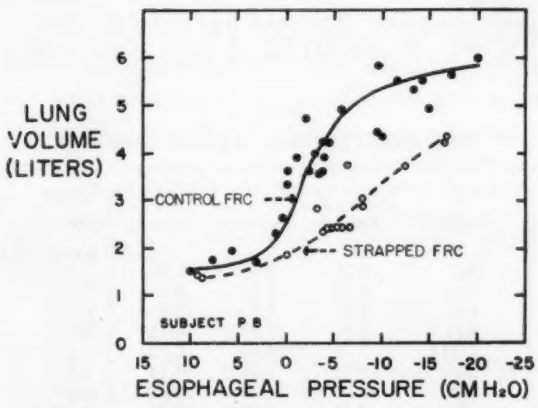



FIG. 3A. GRAPH OF PRESSURE-VOLUME RELATIONSHIP OF THE LUNG, SUBJECT PB. Curve (——) was obtained prior to chest restriction. The pressure-volume relationship was displaced to the right and downward during restriction, curve (- -), i.e., transpulmonary pressure was greater than at equivalent lung volume in the control state.

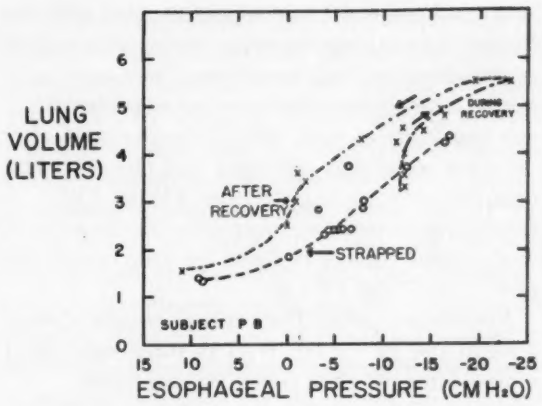



FIG. 3B. CURVE (-·-) WAS OBTAINED AFTER RELEASE OF RESTRICTION. Normal lung pressure-volume relationships were restored via a hysteresis-like pathway (see arrows) following a deep inspiration.

lung compliance did not appear to alter with the change in breathing frequency during either state. A reduction of lung compliance, measured at a resting lung volume corresponding to control FRC, was detectable in most subjects immediately after the chest corset was tightened, and this procedure occupied only about 1 minute (Table IV). A single deep inspiration, taken after the chest corset was released usually restored the lung compliance to normal (Table IV).

*Venous pressure.* The venous pressure showed a mean rise of 1.2 cm $H_2O$ in three subjects, 1 minute after the chest corset was tightened.

TABLE IV

*Onset of and recovery from changes in lung compliance*

| Subject | Lung compliance* | | | | |
|---|---|---|---|---|---|
| | | Chest restricted | | | |
| | Control | At reduced FRC | In inspiratory position† | After restriction released | After deep inspiration |
| | *L/cm $H_2O$* | | | | |
| TS | 0.31 | 0.16 | 0.17 | 0.17 | 0.22 |
| GP | 0.12 | 0.09 | 0.08 | 0.09 | 0.13 |
| AD | 0.21 | 0.13 | 0.12 | 0.22 | 0.29 |
| EL | 0.20 | | 0.15 | 0.16 | 0.22 |
| ST | 0.13 | | 0.15 | 0.11 | 0.13 |
| GL | 0.22 | | 0.19 | 0.20 | 0.29 |
| Mean | 0.198 | | 0.143 | 0.158 | 0.213 |
| SE | 0.028 | | 0.016 | 0.020 | 0.029 |

* Measured during spontaneous breathing.
† At about control FRC immediately after tightening chest corset.

*Airway conductance.* In Figure 4 the curve, together with the standard error of the estimate, relates airway conductance to thoracic gas volume over the range of the vital capacity for ten subjects in the control state. The mean airway conductance for these subjects at resting lung volume is also shown and is designated as "control." It was significant that the average airway conductance in these subjects was not decreased at the reduced resting lung volume caused by chest strapping (see point designated as "strapped," Figure 4, and also Table V), whereas it was decreased during voluntary panting at the same reduced lung volume. This finding was further investigated by making repeated measurements of airway conductance and thoracic gas volume in three subjects who panted continuously for as long as possible without chest strapping, at a lung volume close to residual volume. In the course of about 1 minute the initially low airway conductance in these subjects had risen to about normal.

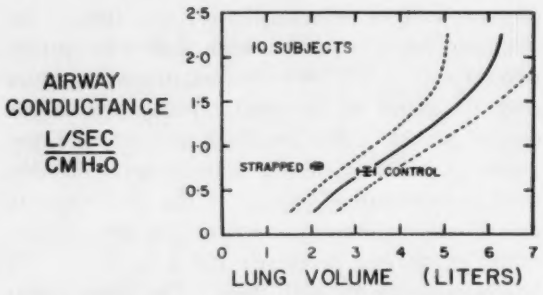



FIG. 4. GRAPH OF AIRWAY CONDUCTANCE AND LUNG VOLUME. Airway conductance was measured in 10 subjects at resting respiratory level prior to chest restriction (control) and at reduced lung volume during chest restriction (strapped). The average value for airway conductance was not significantly changed (Table V); but an equivalent voluntary reduction of lung volume in the same subjects without chest restriction (curve with standard error of estimate) lowered the airway conductance.

*Distribution of inspired gas.* Chest strapping caused a slight but statistically significant increase in the non-uniformity of alveolar ventilation as measured by the nitrogen meter single breath test (Table VI).

*Underventilated gas within the lungs.* When a deep inspiration of oxygen was taken by Subjects TS, PS, GL and AD during the measurement of

TABLE V

*Effect of chest restriction on airway conductance*

| Subject | FRC | | Airway conductance | |
|---|---|---|---|---|
| | Control | Restricted | Control | Restricted |
| | *L* | | *L/sec/cm $H_2O$* | |
| SS | 3.78 | 2.62 | 1.43 | 1.17 |
| NS | 3.25 | 2.74 | 0.43 | 0.68 |
| CC | 3.37 | 2.15 | 0.54 | 0.53 |
| AD | 4.55 | 2.60 | 0.78 | 0.87 |
| DM* | 2.40 | 1.61 | 0.72 | 1.14 |
| GL | 3.42 | 2.35 | 0.74 | 0.79 |
| GP | 2.20 | 1.52 | 0.45 | 0.60 |
| HL | 3.95 | 2.07 | 0.74 | 0.43 |
| PB | 3.01 | 1.95 | 0.65 | 0.60 |
| DM* | 2.52 | 1.74 | 0.84 | 1.02 |
| KA | 3.20 | 1.98 | 0.49 | 0.67 |
| Mean | 3.24 | 2.21 | 0.71 | 0.77 |
| SE | 0.21 | 0.13 | 0.08 | 0.08 |

* Studies performed on two occasions on this subject.

TABLE VI

*Chest restriction and uniformity of alveolar ventilation*

| Subject | Alveolar gas uniformity* index | |
|---|---|---|
| | Control | Restricted |
| SS | 0.4 | 1.7 |
| GL | 0.1 | 2.3 |
| GP | 1.1 | 1.7 |
| HL | 0.7 | 0.7 |
| DM† | 0.5 | 0.7 |
| DM† | 0.0 | 1.1 |
| PB | 0.3 | 1.1 |
| AD | 1.1 | 2.8 |
| NS | 0.0 | 0.7 |
| JB | 0.4 | 1.4 |
| Mean | 0.5 | 1.4 |
| SE | 0.13 | 0.26 |
| | | p = 0.001 |

* Per cent $N_2$ increase during expiration of 750 to 1,250 ml following a single breath of $O_2$. Mean of 3 determinations.

† Studies on same subject, 2 occasions.

a nitrogen dilution curve in the control state, the concentration of nitrogen fell in the succeeding expiration to a predictable level when the end-expiratory nitrogen concentration was plotted against the cumulative volume of oxygen inspired. However, when this experiment was repeated in Subjects TS, PS, GL, AD and CC, whose chests were strapped, the concentration of nitrogen in the succeeding expiration was elevated (Figure 5). The additional nitrogen had apparently been released from poorly ventilated regions of the lung and its volume was calculated by measuring the area beneath the elevation in the nitrogen dilution curve. The mean volume of nitrogen released was 6 ml, range 0 to 15 ml.

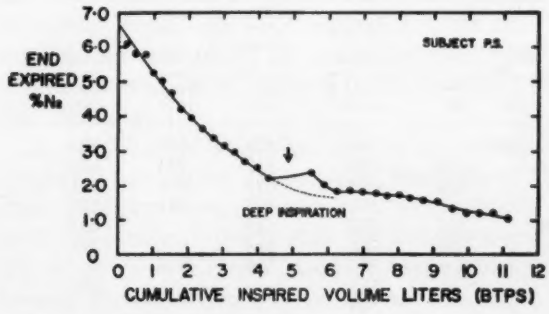



FIG. 5. GRAPH OF PORTION OF $N_2$ DILUTION CURVE DURING CHEST RESTRICTION (ARBITRARY ZERO ON ABSCISSA). A deep inspiration of $O_2$ with the chest restricted caused an elevation of end-expired per cent $N_2$.

In other experiments the chest corset was released during the course of the nitrogen dilution procedure (Figure 6). This allowed the subject's FRC to return to normal and the end-expiratory nitrogen concentration rose during several subsequent tidal breaths with the elimination of additional nitrogen. The mean volume released was 37 ml, range 7 to 90 ml. During succeeding tidal breaths (Figure 6) the expired nitrogen concentration again fell in a predictable manner. However, a deep inspiration caused a further elevation in the end-expired nitrogen concentration, indicating the elimination of a further volume of nitrogen (mean volume 8 ml, range 4 to 12 ml).

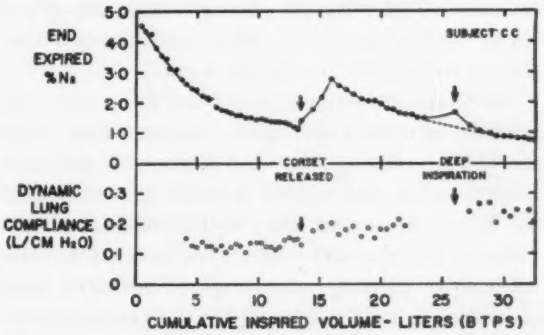



FIG. 6. UPPER DIAGRAM: GRAPH OF PORTION OF A NITROGEN DILUTION CURVE. End-expired per cent $N_2$ and cumulative inspired volume of $O_2$ (arbitrary zero) during and after release of chest restriction. End-expired per cent $N_2$ rose following release of corset and again following a deep inspiration of $O_2$. LOWER DIAGRAM: Lung compliance during spontaneous breathing, same experiment. Lung compliance rose after release of corset, permitting return of lung volume to about control functional residual capacity. Lung compliance was further increased (to normal value for this subject) by deep inspiration of $O_2$.

It has previously been shown that the altered pressure-volume relationship of the lung was restored to normal by a deep inspiration once the chest strapping was released (Figure 3 and Table IV). The lower diagram in Figure 6 shows that normal lung compliance was restored at the same time that nitrogen was released by the deep inspiration. Similar findings were obtained in the other subjects. In a final series of experiments the deep inspiration was delayed until the three subjects, TS, GL and CC had continued to

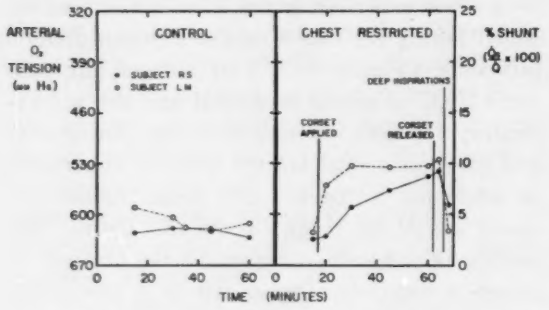



FIG. 7. ARTERIAL OXYGEN TENSION DURING A ONE HOUR PERIOD BREATHING 100 PER CENT OXYGEN. Control (left) and alterations following tight application of a chest corset (right) showing increased percentage of venous admixture. Recovery on release of chest corset.

breathe oxygen for 7 minutes after the chest corset was released. No additional nitrogen was then detectable in any of the three.

As already mentioned, the quantity of nitrogen released in these experiments was measured from the curve relating the end-expiratory nitrogen concentration and minute volume of ventilation. To determine the accuracy of this method of calculation the measurements were checked in one subject by plotting mean expired nitrogen concentration, obtained by graphic integration of the instantaneous expired nitrogen concentration and volume, against minute volume. Using this more laborious method of calculation, reasonable agreement was found.

*Systemic arterial oxygen tension.* The arterial oxygen tension of two subjects, LM and RS, was relatively constant when drawn at intervals during a 1 hour control period of oxygen breathing. However, when the chest corset was tightened in subjects who had already breathed oxygen for 15 minutes, a fall of arterial oxygen tension was detectable 5 to 45 minutes later in six out of eight subjects (Figure 7). Following release of the chest corset the arterial oxygen tension continued to be decreased until one or more deep inspirations of oxygen were taken. One subject became faint after 15 minutes of chest restriction. His arterial oxygen tension had not decreased so that his symptoms could not be attributed to arterial hypoxemia. The arterial $P_{CO_2}$ was measured in one of the subjects (DJ, Table VII) and found to be unchanged from the control value of 44 mm Hg, after chest strapping. In two other subjects the end-expired alveolar $CO_2$ concentration was measured with a $CO_2$ analyzer and was not altered by chest restriction.

## DISCUSSION

In these experiments, chest restriction led to a change in the pressure-volume relationship of the lungs in normal man. There was slightly uneven distribution of inspired gas, measured by the single breath test, and a small quantity of very poorly ventilated gas measured by nitrogen washout procedures. Airway resistance was not increased.

TABLE VII

*Systemic arterial oxygen tension in subjects breathing 100 per cent oxygen with chest restriction*

| | Arterial oxygen tension (mm Hg) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | During chest restriction (minutes after chest restricted) | | | | | | | | | | |
| Subject | Before chest restriction (after 15 min $O_2$ breathing) | 1 | 5 | 10 | 15 | 30 | 40 | 45 | Immediately pre-release of corset | 1 Min after release but before deep inspirations | After 1 deep inspiration | After several deep inspirations |
| GJ | 605* (606, 604) | 585 | 586 | | 591 | 586 | 569 | | 569 | 554 | 578 | 608 |
| JJ | 605* (605, 605) | 573 | 556 | | 546 | 532 | | 536 | 536 | 517 | 593 | 605 |
| EL | 605* (603, 606) | 603 | | 606 | 613 | 626 | | 623 | 623 | 623 | 611 | 611 |
| ER† | 592* (592, 592) | 598 | 590 | | 591 | | | | 591 | 590 | 584 | 572 |
| LM | 627 | | 564 | | 537 | 538 | | 537 | 537 | 529 | | 625 |
| RS | 635* (634, 636) | 639 | 631 | | 594 | 570 | | 551 | 551 | 543 | | 591 |
| LU | 607 | 593 | 604 | | 605 | 575 | | | 575 | 559 | 568 | |
| DJ‡ | 597 | | | | | 551 | | | 551 | | | 616 |
| Mean | 609 | | | | | | | | 569 | | | |
| SE | 5.1 | | | | | | | | 11.8 | | | |

* Mean of duplicate arterial samples.
† Corset was released after 15 min, because of faintness of subject.
‡ Measured supine during cardiac catheterization. Normal heart and lungs by physiological study.

A slight increase in venous admixture to arterial blood was detected. These effects were reversed when the subjects took a deep breath after the release of chest restriction.

We believe we have shown that forces external to the chest cage, by reducing thoracic gas volume, can affect the function of the lungs. We speculate that this effect may occur in other conditions in which there is a reduction of thoracic gas volume, as in individuals wearing tight garments, or perhaps aviation suits designed to counteract gravitational stress. Reduced thoracic gas volume may occur if the chest is strapped for rib fracture, encased in a body cast, or is subjected to hydrostatic pressure under water or to increased gravitational force. The total lung capacity may be reduced in clinical disorders that affect the expansion of the chest wall or pleura: *1*) paralysis of the respiratory muscles; *2*) flail chest resulting from multiple rib fractures; *3*) thoracic deformity in kyphosis or scoliosis; *4*) funnel chest; *5*) thoracoplasty; *6*) ankylosing spondylitis; *7*) obesity; *8*) fibrothorax or calcification of the pleura. A complete discussion of the alterations of pulmonary function in these conditions is beyond the scope of this paper. The reader is referred to other articles on the subject (16–21).

In young patients with scoliosis (19, 21), one frequently finds that the static lung volumes are decreased, lung compliance is reduced, there is slight abnormality of the distribution of inspired gas and, unexpectedly (21), the airway conductance may be normal despite the reduction of thoracic gas volume. There is similarity between these findings and those that we produced in normal subjects by acute experimental restriction of chest cage expansion. We suggest that the pulmonary changes found in young patients with scoliosis may be secondary to a reduction of thoracic gas volume.

In searching for an explanation for the altered mechanical properties of the lung that followed chest strapping several possibilities were considered. It seemed unlikely that the changes were the result of pulmonary vascular engorgement, because the increase in venous pressure was slight in contrast to that obtained under different conditions (22). It seemed more likely that there had been an alteration of the number of terminal lung units (a terminal lung unit being thought to consist of an airway, small airspace, alveolar walls and capillaries) participating in expansion and ventilation, or an alteration of the elastic characteristics of the lung tissue or surface lining film. The following part of the discussion is a further attempt to analyze these possibilities in the light of our experimental data, compared with the findings of other investigators.

An observation which pointed toward a change in the number of ventilated lung units was the release of nitrogen in association with the recovery of the normal pressure-volume relationship of the lung.

After chest strapping the single breath test for distribution of gas was almost unchanged, and lung compliance was not diminished by increasing the breathing frequency. We concluded that the accessible portions of the lung were evenly ventilated, and time constants were not uneven throughout this region (23). Hence the release of nitrogen from the lung following an increase of resting lung volume or with a deep inspiration seemingly indicated that the gas had been present in virtually nonventilated regions. The smallness of the volume of nitrogen released makes it unlikely that closure of major (e.g., lobar or segmental) airways occurred, and we are inclined to believe that closure had occurred in terminal lung units. A mechanical basis for the closure and opening of such units has been described (1, 24) and the phenomenon of their sequential opening has been observed in isolated animal lungs (25). Our data do not permit conclusions to be drawn on the volume of gas that might be trapped in an occluded lung unit (24, 26) or on the number of units that were occluded.

Despite the reduction of the resting lung volume (11) and the apparent closure of lung units and therefore reduction of the total number of conducting airways, mean airway conductance was not decreased with chest restriction. Furthermore, it rose toward normal during continued voluntary panting in the expiratory position. The explanation of these apparent paradoxes appears to be enlargement of the caliber of the patent airways by the increased lung elastic pressure (27).

It would seem that some of the lung units that had become occluded at small lung volume temporarily remained so during tidal breathing after return, unstrapped, to resting lung volume. We

failed, however, to detect any evidence (nitrogen release) of the existence of occluded units in subjects at normal resting lung volume without previous restriction. This is consistent with the findings of others (28) and with the observation that the volume of the functional residual capacity was no different in normal subjects, whether measured plethysmographically or by a nitrogen dilution technique (29). The hysteresis, i.e., failure of the lung to retrace the same pressure-volume curve on inflation and deflation, normally detectable on taking a deep inspiration starting from resting lung volume (1, 30), may therefore be due to mechanisms other than the recruitment of lung units, such as changes in the surface lining film or in tissues of the lung.

The fall in systemic arterial oxygen tension during chest restriction is of theoretical interest. It could be due to the shunting of blood through nonventilated regions of the lung, to a fall in the oxygen tension of the mixed venous blood, or to a decrease in alveolar oxygen tension. Since the subjects had been breathing oxygen for 15 minutes prior to chest restriction, the alveolar oxygen tension would be equal to the atmospheric pressure minus the partial pressures of water vapor and of carbon dioxide. Our evidence suggests that there was no alteration in the arterial or alveolar $P_{CO_2}$ despite the slight increase of respiratory frequency and decrease of tidal volume that occurred during chest restriction. Further studies will be needed, however, to distinguish between the two remaining possibilities.

As a physiological technique, chest restriction permits measurements to be made at equal lung volume but at different transpulmonary pressures, thus allowing separation of the effects of volume and pressure. This principle has been applied to the study of pulmonary hemodynamics in anesthetized dogs (31) and may be applicable in unanesthetized man.

## SUMMARY

1. The total lung capacity and its subdivisions were reduced by restricting chest expansion in normal man.

2. The lung pressure-volume relationship was altered (smaller volume resulting from unit pressure) over much of the vital capacity. Respiratory frequency was increased and tidal volume was reduced. Airway conductance at resting lung volume was not changed despite reduced functional residual capacity. There was slight unevenness of alveolar ventilation with evidence of non- or poorly ventilated lung units. Systemic arterial $O_2$ tension fell during oxygen breathing, and there was a slight increase of peripheral venous pressure.

3. Following release of chest restriction, the mechanical changes in the lungs were reversed via a hysteresis-like pathway. They appear to be explained in part by the opening of lung units which had become closed during chest restriction.

## ACKNOWLEDGMENTS

The authors wish to express their indebtedness to Dr. George Polgar for the estimations of arterial oxygen tension and to Drs. D. H. Lewis and M. Sackner of the Heart Station, Philadelphia General Hospital, for permitting the study of a patient during cardiac catheterization.

## REFERENCES

1. Mead, J., Whittenberger, J. L., and Radford, E. P., Jr. Surface tension as a factor in pulmonary volume-pressure hysteresis. J. appl. Physiol. 1957, **10**, 191.
2. Ferris, B. G., Jr., and Pollard, D. S. Effect of deep and quiet breathing on pulmonary compliance in man. J. clin. Invest. 1960, **39**, 143.
3. Haldane, J. S., Meakins, J. C., and Priestley, J. G. The effects of shallow breathing. J. Physiol. (Lond.) 1919, **52**, 433.
4. D'Silva, J. L., Freeland, D. E., and Kazantzis, G. The performance of patients with ankylosing spondylitis in the maximum ventilatory capacity test. Thorax 1953, **8**, 303.
5. Carton, R. W., and Sepp, E. On taping the chest. Amer. Rev. Tuberc. 1957, **76**, 167.
6. DuBois, A. B., Botelho, S. Y., Bedell, G. N., Marshall, R., and Comroe, J. H., Jr. A rapid plethysmographic method for measuring thoracic gas volume: A comparison with a nitrogen washout method for measuring functional residual capacity in normal subjects. J. clin. Invest. 1956, **35**, 322.
7. Comroe, J. H., Jr., and Fowler, W. S. Lung function studies: VI. Detection of uneven alveolar ventilation during a single breath of oxygen; a new test of pulmonary disease. Amer. J. Med. 1951, **10**, 408.
8. Mead, J., and Whittenberger, J. L. Physical properties of human lungs measured during spontaneous respiration. J. appl. Physiol. 1953, **5**, 779.

9. Marshall, R., and DuBois, A. B. The measurement of the viscous resistance of the lung tissues in normal man. Clin. Sci. 1956, **15**, 161.
10. DuBois, A. B., Botelho, S. Y., and Comroe, J. H., Jr. A new method for measuring airway resistance in man using a body plethysmograph: Values in normal subjects and in patients with respiratory disease. J. clin. Invest. 1956, **35**, 327.
11. Briscoe, W. A., and DuBois, A. B. The relationship between airway resistance, airway conductance and lung volume in subjects of different age and body size. J. clin. Invest. 1958, **37**, 1279.
12. Lewis, B. M., Forster, R. E., and Beckman, E. L. Effect of inflation of a pressure suit on pulmonary diffusing capacity in man. J. appl. Physiol. 1958, **12**, 57.
13. Polgar, G., and Forster, R. E. Measurement of $Po_2$ in small unstirred blood samples with a Clark electrode covered with a relatively impermeable membrane. Fed. Proc. 1959, **18**, 433.
14. Caro, C. G., Butler, J., and DuBois, A. B. Effects of chest strapping on lung mechanics. Physiologist 1958, **1** (no. 4), 8.
15. McIlroy, M. B., Butler, J., and Finley, T. Effect of chest strapping on pulmonary function in normal subjects. Fed. Proc. 1959, **18**, 102.
16. Chapman, E. M., Dill, D. B., and Graybiel, A. The decrease in functional capacity of the lungs and heart resulting from deformities of the chest: Pulmonocardiac failure. Medicine (Baltimore) 1939, **18**, 167.
17. Ferris, B. G., Jr., Mead, J., Whittenberger, J. L., and Saxton, G. A., Jr. Pulmonary function in convalescent poliomyelitic patients, III. Compliance of the lungs and thorax. New Engl. J. Med. 1952, **247**, 390.
18. Fishman, A. P., Turino, G. M., and Bergofsky, E. H. The syndrome of alveolar hypoventilation (Editorial). Amer. J. Med. 1957, **23**, 333.
19. Bergofsky, E. H., Turino, G. M., and Fishman, A. P. Cardiorespiratory failure in kyphoscoliosis. Medicine (Baltimore) 1959, **38**, 263.
20. Hanley, T., Platts, M. M., Clifton, M., and Morris, T. L. Heart failure of the hunchback. Quart. J. Med. 1958, **27**, 155.
21. Caro, C. G., and Gucker, T., III. Effects of kyphoscoliosis upon mechanics of breathing in children (abstract). Amer. Rev. Tuberc. 1958, **78**, 326.
22. Bondurant, S., Hickam, J. B., and Isley, J. K. Pulmonary and circulatory effect of acute pulmonary effects of acute pulmonary vascular engorgement in normal subjects. J. clin. Invest. 1957, **36**, 59.
23. Otis, A. B., McKerrow, C. B., Bartlett, R. A., Mead, J., McIlroy, M. B., Selverstone, N. J., and Radford, E. P., Jr. Mechanical factors in distribution of pulmonary ventilation. J. appl. Physiol. 1956, **8**, 427.
24. Clements, J. A., Brown, E. S., and Johnson, R. P. Pulmonary surface tension and the mucus lining of the lungs: Some theoretical considerations. J. appl. Physiol. 1958, **12**, 262.
25. Radford, E. P., Jr., and McLaughlin, M. Dependence of lung mechanical properties on anatomic relationships within terminal lung units. Fed. Proc. 1956, **15**, 147.
26. Mead, J., and Collier, C. R. Pulmonary mechanical changes in supine anesthetized dogs (abstract). Amer. J. Physiol. 1956, **187**, 616.
27. Butler, J., Caro, C. G., and DuBois, A. B. Lung tissue tension and airway conductance. Physiologist 1958, 1 (no. 4) 7.
28. Armitage, G. H., and Arnott, W. M. Air distribution in lungs during hyperventilation. J. Physiol. (Lond.) 1949, **109**, 70.
29. Bedell, G. N., Marshall, R., DuBois, A. B., and Comroe, J. H., Jr. Plethysmographic determination of the volume of gas trapped in the lungs. J. clin. Invest. 1956, **35**, 664.
30. Butler, J., White, H. C., and Arnott, W. M. The pulmonary compliance in normal subjects. Clin. Sci. 1957, **16**, 709.
31. Frank, R., Borst, H., Berglund, E., Collier, C., and Whittenberger, J. L. Influence of lung volume and airway pressure on pulmonary vascular resistance in dogs' lungs. Fed. Proc. 1958, **17**, 48.

**SPECIAL NOTICE TO SUBSCRIBERS**

Post Offices will no longer forward the Journal when you move.

Please notify The Journal of Clinical Investigation, Business Office, 333 Cedar Street, New Haven 11, Conn., at once when you have a change of address, and do not omit the zone number if there is one.

# PHYSIOLOGICAL FACTORS AFFECTING AIRWAY RESISTANCE IN NORMAL SUBJECTS AND IN PATIENTS WITH OBSTRUCTIVE RESPIRATORY DISEASE *

By JOHN BUTLER,† COLIN G. CARO, RAPHAEL ALCALA ‡ AND ARTHUR B. DuBOIS §

*(From the Department of Physiology of the Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.)*

(Submitted for publication October 14, 1959; accepted December 4, 1959)

Although the airway conductance [1] increases at larger lung volumes in normal subjects (1), it is reduced in patients with asthma or emphysema who are breathing with a large functional residual capacity (FRC) (2). We have therefore tried to assess the influence of other factors which might affect the airway conductance in both normal subjects and in patients with asthma, bronchitis and emphysema. These include the interrelationship of lung volume, lung elastic pressure and airway conductance, the effect of exercise, forced breathing and normal aging, and the composition of the respired gas and bronchomotor drugs. We investigated the balance of forces regulating the diameter of the airway lumen, i.e., the tone of the airway wall, directed inward, opposed by the traction of the lung tissues, directed outward. We studied whether, in normal subjects, this balance was altered by bronchmotor drugs or by a change in lung elastic pressure following chest strapping, and whether in patients with asthma, the airway conductance/lung volume relationship was altered after forced breathing or exercise. Patterns of disturbance leading to air trapping were studied in patients who had emphysema or bronchitis of toxic etiology.

## METHODS

Airway resistance and thoracic gas volume were measured during panting [2] using an upright body plethysmograph (2). The methods hinge on Boyle's law for compression of gases. Volume change owing to compression of gas inside the thorax is measured by the equal and opposite small displacement of gas around the body. After appropriate calibrations, the intrathoracic gas volume changes found when rebreathing the air of the chamber through a heated flowmeter are converted mathematically to alveolar pressure changes, thence to airway resistance and airway conductance; or, when breathing against a closed shutter and manometer, to thoracic gas volume. In practice, these values are read directly from a precalibrated scale and protractor on the face of an oscilloscope, thus reducing labor. Esophageal pressure and mouth pressures were measured alternately using a capacitance manometer and Brush recorder. Lung elastic pressure (3) at each lung volume was measured as mean esophageal pressure,[3] while panting (4). Chest constriction was accomplished by binding the subject's chest or abdomen, in the expiratory position, with adhesive tape or a long strip of rubber (4). Other tests of pulmonary function were performed according to generally accepted methods. All measurements were made with the subject in the sitting position. Lung volumes are expressed at BTPS.

## RESULTS ON HEALTHY SUBJECTS

*Spontaneous variation of airway resistance with time.* The airway resistance of six healthy subjects was studied at 20 minute intervals over a 2 hour period. Measurements were made during

* Supported in part by a contract between the University of Pennsylvania and the Army Chemical Center Medical Laboratories.

† Rockefeller Traveling Fellow in Medicine. Present address: Department of Medicine, University of Manchester, Manchester, England.

‡ Fellow of the Doherty Charitable Foundation, New York, N. Y. Present address: Clinica de la Concepcion, Madrid, Spain.

§ Established Investigator of the American Heart Association.

[1] Airway conductance, the reciprocal of airway resistance, is defined as the rate of airflow at the mouth for unit pressure difference between alveolus and mouth. It was measured over the range of 0 to 0.5 L per second of inspiratory airflow.

[2] The larynx was maintained wide open while panting at different lung volumes as was seen in two normal subjects who were investigated by indirect laryngoscopy.

[3] Lung elastic pressure is equivalent to static intrapleural pressure and represents the pressure derived from the elastic properties of the lung.

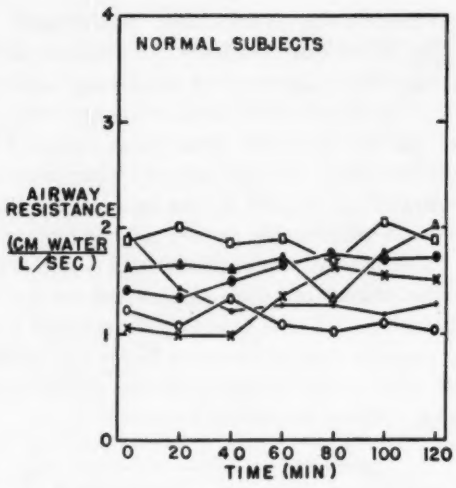



FIG. 1. VARIATION OF AIRWAY RESISTANCE WITH TIME.

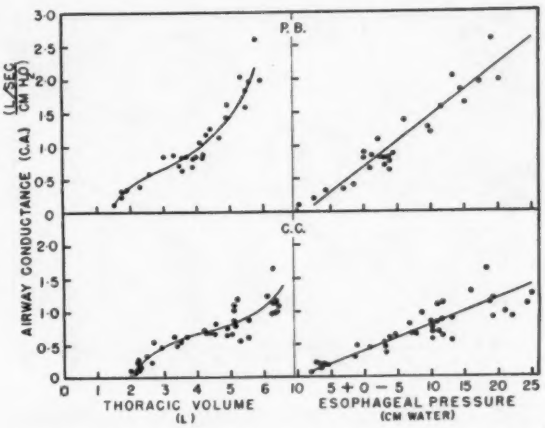



FIG. 2. RELATIONSHIP OF AIRWAY CONDUCTANCE TO THORACIC GAS VOLUME AND ESOPHAGEAL PRESSURE.

panting at about the FRC. The mean value at each sitting was calculated from five consecutive readings. The airway resistance changed but little during the two hours (Figure 1).

*Values for airway resistance in old age.* Airway resistance measured in ten healthy colored subjects, ranging in age from 75 to 90 years, was similar to that of younger people. These subjects had a reduction of maximal breathing capacity, similar to that found by others, and of maximal flow rates. Reduction in the maximum pressure generated by the respiratory muscles and increased lung tissue resistance probably contributed to the diminution of maximal flow rates. Values are listed in Table I.

TABLE I

*Mechanics of breathing in ten normal subjects 75 to 90 years of age* *

| | Average, young adults | Values in older subjects | | Units |
|---|---|---|---|---|
| | | Mean | Range | |
| Total pulmonary resistance, panting | | 2.0 | 1.1–3.2 | cm $H_2O$/L/sec |
| Airway resistance, panting | 0.6–2.4 | 1.3 | 0.7–2.1 | cm $H_2O$/L/sec |
| Pulmonary tissue resistance | 0.0–0.4 | 0.7 | 0.03–1.9 | cm $H_2O$/L/sec |
| Pulmonary compliance, quiet breathing | 0.17 | 0.12 | 0.08–0.20 | L/cm $H_2O$ |
| Airway conductance per unit lung volume (measured at resting lung volume) | 0.13–0.34 | 0.29 | 0.12–0.37 | L/sec/cm $H_2O$/L |
| Pulmonary compliance per unit FRC | 0.038–0.070 | 0.045 | 0.021–0.072 | L/cm $H_2O$/L |
| Maximal expiratory flow rate | 400–500 | 132 | 50–250 | L/min |
| Maximal inspiratory flow rate | 300–500 | 135 | 60–300 | L/min |
| Maximal voluntary mouth pressure | | | | |
| Expiratory: Males | 54–115 | 58 | 38–80 | cm $H_2O$ |
| Females | 57 | 32 | 22–47 | cm $H_2O$ |
| Inspiratory: Males | 60–90 | 33 | 26–36 | cm $H_2O$ |
| Females | 44 | 18 | 8–25 | cm $H_2O$ |

* Resistances were measured over the range 0 to 0.5 L/sec of inspiratory airflow. Maximal flow rates were measured between 0.2 and 1.2 L of inspired or expired volume. Ranges have been given, in preference to standard deviation, because of skew distribution about the mean. Maximal voluntary mouth pressures were measured at a volume close to FRC. A small leak was present in the occluded airway to eliminate effects due to the oropharyngeal muscles acting with a closed glottis.

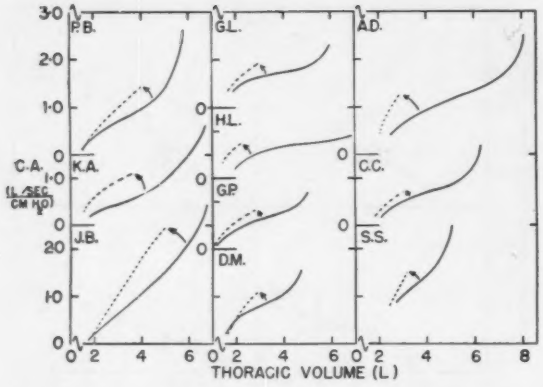



FIG. 3. EFFECT OF CHEST STRAPPING ON RELATIONSHIP OF CONDUCTANCE TO VOLUME. Solid line before, broken line after, chest strapping. A shift in the conductance volume line was observed in each of 10 subjects.

*Lung elastic pressure and airway conductance; the effect of chest strapping.* Airway conductance generally showed a curvilinear relationship to thoracic gas volume (Figure 2, left). Replotting the same airway conductance values versus mean esophageal pressure appeared to yield a straighter line (Figure 2, right).

A change of the pressure-volume curve of the lungs resulted from tightly strapping the chest or abdomen (4); esophageal pressure was more negative at any lung volume above the residual volume (RV). Thus, at the same lung volumes, the lung elastic pressure was greater after chest restriction than in the control state. At each lung volume, airway conductance was also greater after chest strapping than it had been before (Figure 3). When airway conductance was related to lung elastic pressure (Figure 4), measured as mean esophageal pressure at each lung volume, the curves obtained after chest strapping were not on the average different from those obtained in the control state. During recovery after removal of the strapping (Figure 5) the lung elastic pressure-volume relationship formed a large hysteresis loop. The airway conductance-lung volume relationship passed through a similar loop on the return to normal. These results indicated that lung elastic pressure was the common factor responsible for the relationship between airway conductance and lung volume previously reported (1).

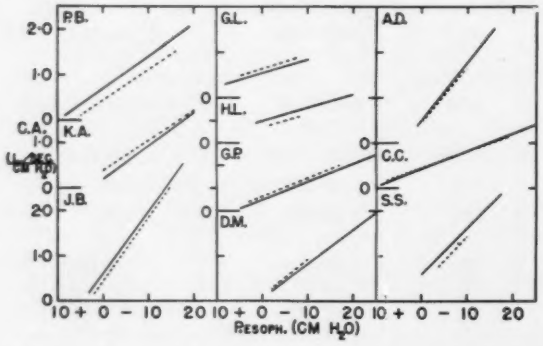



FIG. 4. EFFECT OF CHEST STRAPPING ON RELATIONSHIP OF CONDUCTANCE TO ESOPHAGEAL PRESSURE. Solid line before, broken line after, chest strapping. No average change in the conductance-pressure plot was observed.

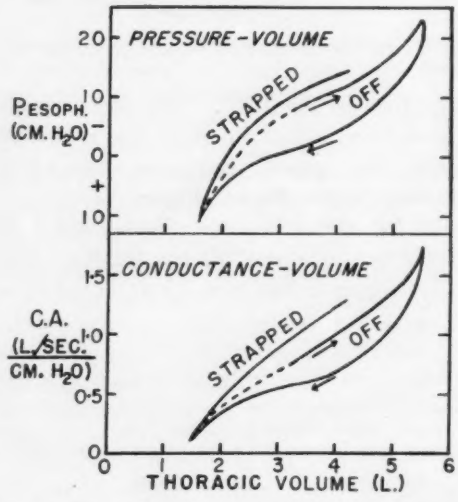



FIG. 5. RELATIONSHIPS DURING FIRST INSPIRATION AFTER REMOVAL OF STRAPPING. Pressure volume (top) and conductance volume (bottom) plots followed hysteresis loops similar in appearance.

*Effect of exercise or forced breathing.* Data obtained before and after exercise or forced breathing are presented in Table II. After exercise the subjects breathed in a slightly more inspiratory position, thereby slightly reducing the airway resistance (1). Forced breaths, consisting of vital capacity and maximal flow rate determinations, failed to change the airway resistance of normal subjects.

*Effect of oxygen or carbon dioxide.* Oxygen administered by a closely fitting, airtight face mask failed to produce any change in airway resistance whether it was measured immediately after the onset of oxygen breathing or at inter-

TABLE II

*Factors which do not appear to affect airway resistance: $O_2$, $CO_2$, water aerosol, exercise, and forced breathing*

| Condition | Subjects | Control period | | Experimental period | | Change in resistance | |
|---|---|---|---|---|---|---|---|
| | | Airway resist. | Volume | Airway resist. | Volume | Mean difference | SE of mean difference |
| | *no.* | *cm $H_2O$/L/sec* | *L* | *cm $H_2O$/L/sec* | *L* | *cm $H_2O$/L/sec* | *cm $H_2O$/L/sec* |
| Rebreathing air or $O_2$ in a bag | 5 | 1.63 | 3.8 | 1.58 | 3.9 | −0.05 | 0.04 |
| Breathing $O_2$ 5 or 6 hours | 9 | 1.61 | 4.4 | 1.62 | 4.6 | 0.006 | 0.06 |
| Rebreathing 7.5 to 10% $CO_2$ in a bag | 7 | 1.39 | 4.3 | 1.38 | 4.3 | −0.01 | 0.06 |
| Breathing 4 to 6% $CO_2$ 1 to 10 min in a chamber | 5 | 1.21 | 4.1 | 1.21 | 4.3 | 0.00 | 0.17 |
| Water aerosol | 4 | 1.52 | 4.1 | 1.54 | 4.0 | 0.02 | 0.05 |
| Exercise | 3 | 1.61 | 3.2 | 1.54 | 3.4 | −0.07 | 0.04 |
| Tests of lung volume and forced breathing | 5 | 1.92 | 2.9 | 1.85 | 2.7 | —0.07 | 0.05 |

vals during an exposure of 5 to 6 hours' duration (Table II). Four to 10 per cent carbon dioxide administered in the respired air over periods of time lasting up to 10 minutes gave variable results, but there was no change in the average airway resistance of the group (Table II).

## RESULTS ON PATIENTS

*The relationship between airway conductance and lung volume in asthma and emphysema.* The airway conductance was measured at different lung volumes in healthy subjects, in symptomatic and asymptomatic asthmatic patients, and in patients with emphysema. Airway conductance was plotted against lung volume and the curve of best fit was drawn by inspection. From the curve, the volume at which airway conductance appeared to approach zero (1) and the airway conductance at the FRC were read. The average slope of the curve of airway conductance versus volume was

TABLE III

*Characteristic alterations in the interrelation of airway conductance and lung volume in patients with asthma and emphysema, and values (in parentheses) following isoproterenol aerosol, 1:200 solution* *

| Subjects | | Airway conductance at FRC | | FRC | | Conduct. change ÷ volume change | | Lung gas volume at zero conductance | |
|---|---|---|---|---|---|---|---|---|---|
| | *no.* | *L/sec/cm $H_2O$* | | *L* | | *L/sec/cm $H_2O$/L* | | *L* | |
| Normal | 5 | 0.60 | (0.77) | 2.6 | (2.6) | 0.28 | (0.50) | 0.8 | (1.0) |
| Symptomless asthma | 4 | 0.38 | | 2.8 | | 0.28 | | 1.5 | |
| Asthma | 3 | 0.19 | (0.49) | 4.8 | (4.1) | 0.09 | (0.23) | 2.7 | (1.9) |
| Emphysema | 5 | 0.18 | (0.36) | 5.0 | (4.6) | 0.16 | (0.29) | 3.8 | (3.4) |

* In addition to these observations, atropine, 0.9 to 1.2 mg, subcutaneously, increased the ratio conductance/volume from 0.27 to 0.48 in four normal subjects; epinephrine, 0.4 ml of a 1:1,000 solution, subcutaneously, changed the conductance from 0.67 (at 3.8 L) to 0.76 (at 3.7 L) in five normal subjects. The FRC's were measured by the plethysmographic method (5).

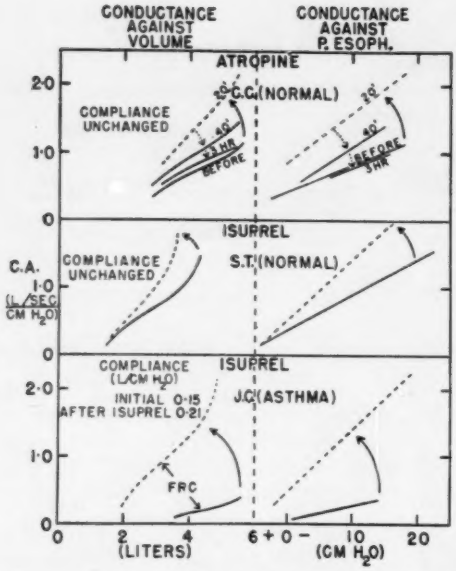



FIG. 6. THE EFFECT OF BRONCHODILATOR DRUGS ON THE RELATIONSHIP BETWEEN AIRWAY CONDUCTANCE AND LUNG VOLUME OR ESOPHAGEAL PRESSURE IN NORMAL SUBJECTS AND IN AN ASTHMATIC PATIENT. Left-hand diagrams relate conductance to volume. Right-hand diagrams relate conductance to esophageal pressure. Solid lines before, and broken lines after, bronchodilator. Time sequence of recovery from atropine shown in upper diagrams.

measured at the FRC. These values are shown in Figure 6 and Table III.

In both the symptomatic asthmatic patients and in the patients with emphysema, the FRC (5) was increased and the airway conductance, measured at the FRC, was reduced. The change in airway conductance per unit change in lung volume was less than normal, and there was an increase of the lung volume at which the extrapolated line of airway conductance approached zero. The failure of the increased FRC of these patients to restore normal airway conductance appeared to be due to the shift in position and lower slope of the airway conductance-lung volume relationship (Figure 6).

*Effect of forced breathing or exercise.* The asthmatic patients who were in symptomatic remission and not on therapeutic drugs had little spontaneous variation of airway resistance. However, after forced breathing, consisting of vital capacity and maximal flow rate determinations, airway resistance increased in the majority. The entire curve of airway conductance against lung volume (Table IV) had shifted. Brief, heavy exercise produced a similar response in two asthmatic subjects.

*Effect of bronchodilator drugs.* Administration of bronchodilator drugs altered the curve of airway conductance versus lung volume in normal subjects, in patients with asthma, and, to some extent, in patients with emphysema (Table III). That this was not attributable to an increase in lung elastic pressure was evident when airway

TABLE IV

*Effect of fast vital capacity tests and exercise on airway conductance in asthmatic patients*

| Procedure and subjects | Airway conductance at FRC | | FRC | | Conduct. change ÷ volume change | | Lung gas volume at zero conductance | |
|---|---|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After | Before | After |
| | *L/sec/cm H₂O* | | *L* | | *L/sec/cm H₂O/L* | | *L* | |
| 5 Normals* | 0.52 | 0.54 | 2.9 | 2.7 | 0.23 | 0.22 | 0.9 | 0.9 |
| VC tests in asthmatics | | | | | | | | |
| ME† | 0.45 | 0.21 | 3.3 | 3.5 | 0.20 | 0.13 | 2.3 | 2.9 |
| JC† | 0.45 | 0.31 | 3.7 | 4.1 | 0.19 | 0.13 | 2.0 | 2.3 |
| LF† | 0.54 | 0.32 | 3.0 | 3.0 | 0.24 | 0.18 | 1.2 | 1.6 |
| MK | 0.74 | 0.65 | 2.4 | 2.4 | 0.29 | 0.30 | 1.0 | 1.0 |
| MC | 0.20 | 0.20 | 3.9 | 4.0 | 0.25 | 0.11 | 2.5 | 3.0 |
| Exercise in asthmatics | | | | | | | | |
| JC† | 0.39 | 0.25 | 2.6 | 2.8 | 0.39 | 0.28 | 1.6 | 2.0 |
| DO† | 0.34 | 0.28 | 3.5 | 4.9 | | | | |

* None of the normal subjects showed any significant change of values after vital capacity tests or exercise.
† These patients showed a significant change. The asthmatic patients, except MC, were asymptomatic at the onset.

TABLE V

*Tests of forced voluntary ventilation and airway resistance in youth, industrial bronchitis, and old age* *

| | Youth | Industrial bronchitis† | Old age |
|---|---|---|---|
| Age | 32 | 43 | 82 |
| Maximal breathing capacity | 150 (8) | 84 (9) | 38 (5) |
| Maximal expiratory flow rate | 476 (19) | 213 (36) | 132 (25) |
| Maximal inspiratory flow rate | 390 (40) | 226 (18) | 135 (25) |
| Airway resistance and % change after bronchodilator | 1.37 (0.26)<br>−29% | 2.94 (0.25)<br>−44% | 1.30 (0.21) |

* Mean values, and standard error (in parentheses).
† Eleven patients were studied. Of these, 6 had accidentally inhaled toxic vapors, and 5 had undergone lengthy industrial exposure to particulate matter (carbon, silica, and iron particles). Other findings in these patients were increased residual volume, uneven ventilation, slight hypoxemia, and gas trapping.

conductance was plotted against esophageal pressure. Figure 6 shows the effect of bronchodilator drugs in the normal and in the asthmatic state.

*Airway resistance and other tests of pulmonary function in patients with chronic bronchitis.* We studied several patients who had been exposed to chemical vapors or particulate matter in the inspired air and who subsequently developed clinical evidence of chronic bronchitis (Table V). These patients had no prior history suggestive of asthma or emphysema. The airway resistance was elevated in most. However, there was an appreciable response to bronchodilator drugs. Values for airflow obtained from tests of forced voluntary ventilation, although depressed, were not so low as is usual in fully developed emphysema. By contrast, normal elderly people had impairment of forced voluntary ventilation but normal airway resistance.

An acute exposure of normal subjects to carbachol aerosol or inert particles dispersed in the inspired air had resulted in an elevation of airway resistance with little or no change in maximal expiratory flow rate (Table VI). At the onset of forced expiration the airways may be widely dilated owing to lung elastic pressure; during forced expiration they tend to collapse from positive intrapleural pressure. We concluded that changes in the state of the airways, while adequately reflected in measurements of airway resistance, were sometimes obscured or erroneously interpreted when forced voluntary ventilation was being used.

TABLE VI

*Response of normal subjects to carbachol aerosol or inert particles* *

| | Before | After |
|---|---|---|
| Airway resistance† | 1.37 (0.26) | 2.63 (0.49) |
| Maximal expiratory flow rate | 476 (19) | 464 (28) |

* Mean values, and standard error (in parentheses).
† These airway resistance values were reported previously in this journal (6). Values for maximal expiratory flow rate reveal that a moderate degree of bronchoconstriction may be overlooked unless suitable methods are used for its measurement.

## DISCUSSION

The airway conductance, measured in the body plethysmograph by relating alveolar pressure to airflow at the mouth, presumably depends upon the patency, number and length of the conducting airways. It remained relatively constant over several hours in normal subjects. Others have shown that the airway conductance increased at larger lung volumes (1); the present results indicate that it varies directly with lung elastic pressures and that its relationship to lung volume is determined by the pressure-volume behavior of the lung. Thus, at the same lung volume there was a rise in airway conductance when the lung elastic pressure was increased following chest constriction. This was presumably due to an enlargement of the airways as a result of the greater pull on their walls and not to a relaxation of the tone in the walls. There was no evidence that the number of patent airways was increased under these conditions; in fact it was more likely that there were fewer, since there was some evidence of gas trapping (7). This effect of lung elastic pressure on airway conductance had been suggested before (8–11) but had not been established experimentally.

Atropine subcutaneously, and isoproterenol by aerosol, increased airway conductance in normal subjects in the absence of any alteration in lung elastic pressure. This suggested that these drugs, by their action in reducing active (tonic) effects in the airway wall, had allowed a greater distention of the airway from the passive (mechanical) effects of the existing lung elastic pressure. However, mucosal shrinkage as well as decrease of tone may be concerned in this rise of airway conductance and our studies do not distinguish between them. Breathing oxygen, carbon dioxide or water aerosol produced no significant change in the airway conductance.

When the airway conductance was reduced by disease (asthma, bronchitis, emphysema) there was less than the normal change in airway conductance from alterations of lung elastic pressure. The distensibility of the airways appeared to be decreased. Furthermore, both the lung elastic pressure and the lung volume at which the airway conductance approached zero, obtained by extrapolation, were increased in these patients. This suggested that gas trapping occurred at greater than normal transpulmonary pressures and lung volumes.

Bronchodilator drugs produced striking results in asthma, a greater-than-expected response in bronchitis of toxic etiology, and some response in emphysema. The relationship of airway conductance to both esophageal pressure and lung volume was altered toward normal by these drugs. They appeared to improve airway distensibility and to reduce the lung volume at which the airway conductance approached zero. However, it is often impossible to distinguish between asthma and emphysema by a therapeutic test with these drugs, since both may respond or be refractory to isoproterenol.

We found that forced breathing or exercise in asthmatic patients may decrease the airway conductance while tending to increase the FRC. This has been suspected clinically but experimental confirmation was lacking. The mechanism of this response is unknown, but it is presumably dependent upon changes in the airway wall, the lung elastic pressure at different volumes being unchanged or increased.

Unsuspected degrees of airway obstruction, uneven ventilation, and arterial oxygen desaturation were found in certain cases of bronchitis of toxic etiology. Maximum flow rates were not so reduced as in emphysema. By contrast, in healthy old age there was a reduction in the maximum flow rates which was not associated with any increase in airway resistance. It appeared to be due to a reduction in the power of the respiratory muscles, the maximum pressure generated at the mouth being reduced in most subjects. There was an increase in the lung tissue resistance of some of these older subjects. Our results may be compared with and extend those of other workers (12, 13).

We have isolated and described some of the factors that affect the balance which we believe to exist between the tension of the airway wall and the transmural pressure gradient imposed by the elastic pull of the lung. However, assuming some degree of regulation of airway patency, we have shown little about the control system. Among other factors still to be explored are the effects of diminished oxygen and $CO_2$ tension, altered pH, lesions of the nervous system, and psychogenic and endocrine factors. Our methods appear to be satisfactory for this type of observation in man.

## SUMMARY

The effects on airway conductance (the reciprocal of airway resistance) of chest strapping, bronchomotor drugs, exercise, forced breathing, oxygen, carbon dioxide, and old age, were measured in normal subjects and compared with similar observations in patients with asthma, bronchitis and emphysema. Airway conductance was found to be dependent upon lung elastic pressure rather than on lung volume. This relationship was altered by bronchomotor drugs. Breathing oxygen and different concentrations of carbon dioxide did not alter the airway resistance. In several patients with mild asthma, airway resistance increased after forced breathing or exercise. In normal subjects, airway conductance appeared to depend upon a balance of forces between the tension in the airway walls and the opposing transmural pressure gradient due to the elastic traction of the lung. The airway conductance was found to be reduced and the balance of forces was altered in patients who had lower airway obstruction.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Julius H. Comroe, Jr., for helpful advice. We acknowledge the collaboration of Comdr. Robert L. Burdick, MC, USN, of the Air Crew Equipment Laboratory, Philadelphia, in the evaluation of the effects of breathing oxygen, and of Drs. W. A. Briscoe and J. Zabara in other parts of this work. We appreciate the cooperation of the ten older normal subjects and the officers of the Stephen Smith Home for the Aged.

## REFERENCES

1. Briscoe, W. A., and DuBois, A. B. The relationship between airway resistance, airway conductance and lung volume in subjects of different age and body size. J. clin. Invest. 1958, **37**, 1279.
2. DuBois, A. B., Botelho, S. Y., and Comroe, J. H., Jr. A new method for measuring airway resistance in man using a body plethysmograph: Values in normal subjects and in patients with respiratory disease. J. clin. Invest. 1956, **35**, 327.
3. Mead, J., and Whittenberger, J. L. Physical properties of human lungs measured during spontaneous respiration. J. appl. Physiol. 1953, **5**, 779.
4. Caro, C. G., Butler, J., and DuBois, A. B. Effects of chest strapping on lung mechanics. Physiologist 1958, **1** (no. 4), 8.
5. DuBois, A. B., Botelho, S. Y., Bedell, G. N., Marshall, R., and Comroe, J. H., Jr. A rapid plethysmographic method for measuring thoracic gas volume: A comparison with a nitrogen washout method for measuring functional residual capacity in normal subjects. J. clin. Invest. 1956, **35**, 322.
6. DuBois, A. B., and Dautrebande, L. Acute effects of breathing inert dust particles and of carbachol aerosol on the mechanical characteristics of the lungs in man. Changes in response after inhaling sympathomimetic aerosols. J. clin. Invest. 1958, **37**, 1746.
7. Caro, C. G. Lung compliance, hysteresis and closure of lung units in man. Fed. Proc. 1959, **18**, 23.
8. Fry, D. L., Ebert, R. V., Stead, W. W., and Brown, C. C. Mechanics of pulmonary ventilation in normal subjects and in patients with emphysema. Amer. J. Med. 1954, **16**, 80.
9. Shepard, R. H., Campbell, E. J. M., Martin, H. B., and Enns, T. Factors affecting the pulmonary dead space as determined by single breath analysis. J. appl. Physiol. 1957, **11**, 241.
10. Martin, H. B., and Proctor, D. F. Pressure-volume measurements on dog bronchi. J. appl. Physiol. 1958, **13**, 337.
11. Butler, J., and Arnott, W. M. The work of pulmonary ventilation at different respiratory levels. Clin. Sci. 1955, **14**, 703.
12. Frank, N. R., Mead, J., Siebens, A. A., and Storey, C. F. Measurements of pulmonary compliance in seventy healthy young adults. J. appl. Physiol. 1956, **9**, 38.
13. Pierce, J. A., and Ebert, R. V. The elastic properties of the lungs in the aged. J. Lab. clin. Med. 1958, **51**, 63.

# INDOCYANINE GREEN: OBSERVATIONS ON ITS PHYSICAL PROPERTIES, PLASMA DECAY, AND HEPATIC EXTRACTION *

By GILBERT R. CHERRICK,† SAMUEL W. STEIN,‡ CARROLL M. LEEVY § AND CHARLES S. DAVIDSON

*(From the Thorndike Memorial Laboratory and the Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.)*

(Submitted for publication August 31, 1959; accepted December 4, 1959)

Indocyanine green [1] (ICG) is a tricarbocyanine dye (Figure 1) which has been used in the indicator dilution technique for measuring cardiac output (1, 2). Animal studies (3) and preliminary observations of human subjects (4, 5) have suggested that this dye may have characteristics that could make its uptake, storage, and excretion by the liver helpful indices of hepatic function. The present investigations were carried out to determine whether indocyanine green has properties that may render it suitable for assessing liver function and hepatic blood flow in man.

## METHODS AND PROCEDURES

*Physical properties.* Indocyanine green was prepared for intravenous administration by dissolving the dye in distilled water to a concentration of 5 mg per ml. Readings of dye concentration were made in a Beckman DU spectrophotometer at 815 mμ.

Volume of distribution of radioiodinated human serum albumin [2] was compared with the initial volume of distribution of ICG in 4 normal subjects. Each subject was given approximately 20 μc of the $I^{131}$-labeled albumin and 50 mg of ICG in a rapid intravenous injection. Venous samples were taken at intervals during the following 12 minute period. Radioactivity was counted in a scintillation well counter. Dye concentration in each sample was plotted on semilogarithmic paper. Extrapolation of the exponential portion of the decay curve back to zero time permitted calculation of initial volume of dye distribution. The radioactivity in samples taken 5 minutes after injection was employed to estimate the volume of distribution of labeled albumin.

Absorption spectra of ICG in a 5 per cent solution of human serum albumin, in normal human plasma, and in distilled water alone, were plotted, using spectrophotometer readings made between 635 and 900 mμ. Starch block electrophoresis was carried out on four 0.5 ml samples of normal human plasma to which ICG had been added in a concentration of 0.25 mg per 100 ml. The block was poured with reagent grade soluble starch which had been washed 4 times with Tris-maleic buffer, pH 8.6. Electrophoresis was continued for 20 hours at 7° C. Whatman no. 3 filter paper was then applied to the surface of the block. When dry, the paper was stained with bromphenol blue and decolorized with water. The starch block was cut into segments corresponding to the location of protein bands on the filter paper. Protein-dye complex was eluted from the starch into Tris-maleic buffer, pH 8.6.

Ascending chromatography of bile which had been obtained from T-tube drainage of a patient and which

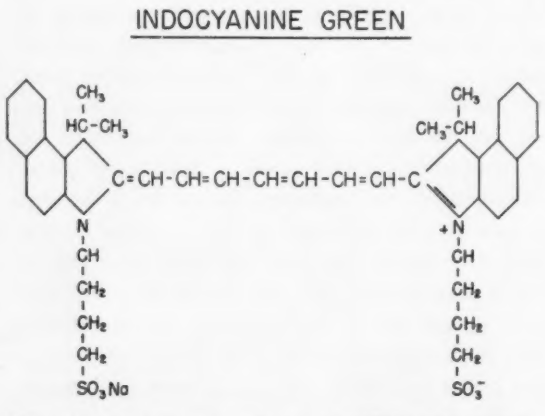



FIG. 1. STRUCTURAL FORMULA OF INDOCYANINE GREEN.

* This investigation was supported in part by the United States Army Medical Research and Development Command, Department of the Army, under Contract No. DA-49-193-MD-2013, and in part by the Life Insurance Medical Research Fund.

† Public Health Service Research Fellow of the National Heart Institute. Present address: Nemazee Hospital, Shiraz Medical Center, Shiraz, Iran.

‡ Research Fellow of the American Heart Association, Inc.

§ Public Health Service Special Research Fellow of the National Institute of Arthritis and Metabolic Diseases. Present address: Seton Hall College of Medicine, Jersey City, N. J.

[1] Cardio-Green; Hynson, Westcott, and Dunning, Baltimore, Maryland.

[2] RISA; Abbott Laboratories, Chicago, Ill.

contained ICG was carried out for 18 hours in 3 systems: 1) butanol: acetic acid: water (10:4:8, vol/vol); 2) methylethyl ketone: propionic acid: water (75:25:30, vol/vol); and 3) pyridine: water: butanol (3:4:2, vol/vol). S and S no. 598 filter paper was used. Four spots were chromatographed with each buffer system: dye in aqueous solution, human bile to which dye had been added, human bile into which dye had been secreted, and a sample of bile containing no dye.

*Site of removal from plasma.* Five patients without evidence of kidney or liver disease were studied to determine whether ICG is ordinarily excreted in the urine. They were given 0.5 mg per kg body weight of ICG intravenously, and total urine collections were made during a 6 hour period following dye administration. Urine was collected in bottles containing 5 ml of a 5 per cent solution of human serum albumin in order to prevent deterioration of dye.

Appearance and disappearance of ICG was studied in bile taken from T-tube drainage of two patients who recently had undergone cholecystectomy for gallstones. Each received 2 mg per kg of dye as a single intravenous dose. Samples of bile were collected at intervals during periods of 6 and 19 hours, respectively. Two drops of a 5 per cent solution of human serum albumin were added to each 5 ml bile sample which was then diluted as necessary with distilled water to produce a dye concentration suitable for spectrophotometric analysis.

Peripheral arteriovenous differences of ICG were studied in one normal subject. He was given a priming dose of 10 mg of ICG intravenously, and a constant infusion of the dye at a rate of about 0.5 mg per minute was administered through a polyethylene catheter inserted into an antecubital vein of the left arm. During the infusion, simultaneous sampling was done at 5-minute intervals from a Cournand needle placed in the left brachial artery and from a polyethylene catheter inserted into a right antecubital vein.

*Plasma decay.* Plasma decay of ICG and sulfobromophthalein [3] (BSP) was studied in a group of 9 patients without clinical or laboratory evidence of liver disease (Table I) and in 16 patients with confirmed liver disease (Table II). Fourteen of the latter had cirrhosis, in 8 of whom it was associated with chronic alcoholism and in 6 of unknown etiology. Two patients with cirrhosis had normal liver function tests, but in both, hepatic histology was consistent with the diagnosis. Two patients had infectious hepatitis.

All subjects were given ICG, 0.5 mg per kg body weight, and BSP, 5 mg per kg body weight as single, rapid, intravenous injections at least 2 hours apart. Venous samples were taken at 3 and 5 minutes from the time of dye injection and at 5-minute intervals thereafter for at least 1 hour and in some cases for 2 or 3 hours. One patient with infectious hepatitis had 3 determinations of ICG and BSP decay during the acute illness and convalescent period. Indocyanine green was also administered to 7 of the 9 control subjects (Table I) in amounts of 1.0 mg per kg body weight and to 3 additionally in doses of 2.0 mg per kg body weight, sampling being made as stated above. BSP concentration in serum was determined by alkalinization and reading in an Evelyn colorimeter at 580 mμ.

Plasma dye decay rate was calculated from the following formulae (6):

$$R = 1 - d$$

and

$$\log d = \frac{\log C_2 - \log C_1}{t_2 - t_1}$$

where R is the rate of decay per minute, d is the fraction retained per minute, $C_2$ and $C_1$ are concentrations in plasma at two points during the exponential decay period, and $t_2$ and $t_1$ are the times in minutes corresponding to $C_2$ and $C_1$.

*Hepatic extraction.* Hepatic extraction rates of ICG and estimated splanchnic blood flow were determined in 7 subjects without liver disease, using right hepatic vein catheterization. The catheter was advanced to wedge position, then withdrawn just far enough to permit free sampling, in order to minimize reflux from the inferior vena cava. ICG was infused in 0.5 per cent albumin solution with the use of a Bowman pump at a constant rate of from 0.3 to 0.7 mg per minute after an initial intravenous loading dose of 10 to 15 mg had been given.

[3] Bromsulphalein; Hynson, Westcott, and Dunning, Baltimore, Md.

TABLE I

*Clinical and laboratory data on patients without evidence of liver disease*

| Subject | Sex | Age | Diagnosis | Cephalin flocculation | Serum bilirubin |
|---|---|---|---|---|---|
| | | | | | *mg/100 ml* |
| AW | M | 27 | Pneumococcal pneumonia | 0 | 0.8 |
| JW | M | 54 | Peptic ulcer | 1+ | 0.3 |
| BO | M | 49 | Alcoholism | 2+ | 0.7 |
| FW | M | 58 | Pneumococcal pneumonia | 1+ | 1.9 |
| EE | M | 51 | Alcoholism | 0 | 0.2 |
| JT | M | 43 | Alcoholism | 1+ | 0.5 |
| JM | M | 42 | Peptic ulcer | 2+ | 0.7 |
| CF | M | 45 | Alcoholism | 1+ | 0.4 |
| CW | M | 48 | Post-traumatic epilepsy | 0 | 0.6 |

TABLE II

*Clinical and laboratory data on patients with liver disease*

| Subject | Sex | Age | Clinical features* | Clinical diagnosis | Liver histology | Cephalin flocculation | Serum bilirubin |
|---|---|---|---|---|---|---|---|
| | | | | | | | *mg/100 ml* |
| | | | | Cirrhosis | | | |
| JL | M | 49 | H, S | Of the alcoholic | Fibrosis, fat, inflammation | 4+ | 16.8 |
| JS | M | 49 | H, S | Of the alcoholic | | 1+ | 2.1 |
| BB | F | 53 | H | Of the alcoholic | | 1+ | 1.6 |
| AB | F | 59 | H, A, S | Of the alcoholic | Fibrosis, alc. hyaline | 3+ | 12.8 |
| ZM | F | 39 | None | Of the alcoholic | Fibrosis, fat | 0 | 0.5 |
| EC | F | 45 | H | Of the alcoholic | Fibrosis, fat | 2+ | 0.4 |
| JM | M | 34 | H | Of the alcoholic | Fibrosis, mild bile stasis | 2+ | 1.8 |
| MS | M | 36 | H, A, S | Of the alcoholic | | 1+ | 1.7 |
| DG | M | 58 | H, S, P-C | Etiology unknown | Portal cirrhosis | 2+ | 1.4 |
| JC | M | 70 | H, S, P-C | Etiology unknown | Postnecrotic cirrhosis | 2+ | 3.9 |
| BM | M | 47 | None | Etiology unknown | | 2+ | 0.6 |
| MM | F | 65 | H, S | Etiology unknown | Fibrosis, fat | 3+ | 0.5 |
| AV | M | 57 | H, S | Etiology unknown | | 2+ | 0.2 |
| RR | M | 48 | None | Etiology unknown | Fibrosis | 1+ | 0.4 |
| RB | M | 32 | H, S | Viral hepatitis | | 4+ | 5.8 |
| EG | M | 29 | | Viral hepatitis | | | |
| | 5-1-59 | | H,S | | | 3+ | 1.0 |
| | 5-8-59 | | None | | | 2+ | 0.6 |
| | 5-18-59 | | None | | | 2+ | 0.4 |

* H = hepatomegaly; S = splenomegaly; A = ascites; P-C = portacaval shunt.

The addition of this amount of albumin had been shown to stabilize dye solutions during the infusion periods. Dye concentration was determined in blood samples taken simultaneously at 10-minute intervals from an indwelling brachial arterial needle and the hepatic vein catheter.

Extraction rate (E.R.) was calculated from the formula:

$$\text{E.R.} = \frac{A - HV}{A}$$

where A is the arterial dye concentration and HV is the dye concentration in hepatic venous blood.

Estimated hepatic blood flow (EHBF) in liters per minute was calculated from the formula:

$$\text{EHBF} = \frac{R}{(P - H)\ \ (1 - HCT)}$$

where R is the total ICG removal rate in milligrams per minute, P is the concentration of ICG in peripheral arterial blood in milligrams per liter, H is the concentration of ICG in hepatic venous blood in milligrams per liter, and HCT is the peripheral venous hematocrit.

Total ICG removal rate (R) was taken as equal to the infusion rate in milligrams per minute during periods when peripheral dye concentration was constant (7).

## RESULTS

*Physical properties.* Initial volume of distribution of indocyanine green, calculated from plasma dye decay curves, and the volume of distribution of radioiodinated albumin at 5 minutes showed close agreement in the four patients studied (Table III). There was close correspondence between the absorption spectrum of ICG in a 5 per cent solution of human serum albumin and in normal human plasma (Figure 2). Absorption maximum was at 815 mμ in both cases. The absorption spectrum of the dye in aqueous solution was distinctly different from the spectra found for the two albumin-containing dye solutions, the absorption maximum being at 790 mμ. On starch block electrophoresis, ICG was found to migrate

TABLE III

*Comparison of volume of distribution of radioiodinated albumin and initial volume of distribution of indocyanine green*

| Subject | $I^{131}$-albumin volume of distribution at 5 min | Initial volume of distribution of ICG |
|---|---|---|
| | *ml* | *ml* |
| JR | 4,202 | 4,351 |
| BO | 3,100 | 2,970 |
| CE | 2,908 | 2,810 |
| JT | 3,636 | 3,801 |

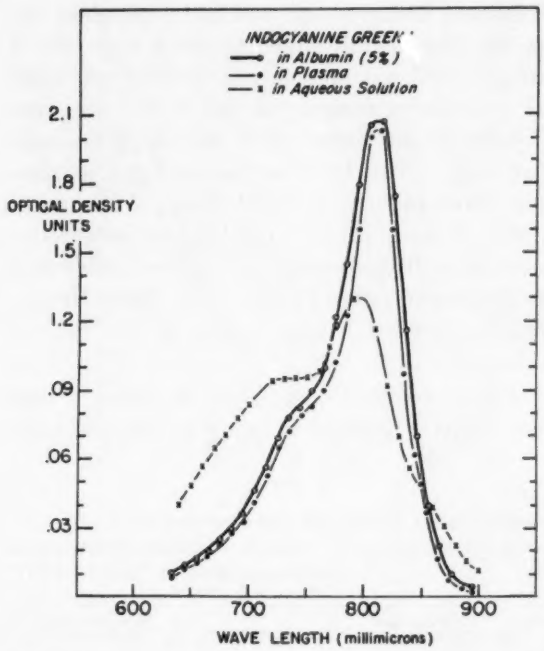



FIG. 2. ABSORPTION SPECTRA OF INDOCYANINE GREEN.

with albumin and to be bound to the other plasma proteins in only small amounts (Table IV). Chromatographic studies carried out in three different systems showed no differences in the rate or pattern of movement of dye in aqueous solution as compared with dye which had been secreted into bile and dye which had been added to bile (Figure 3).

*Site of removal from plasma.* Urine collected from five patients during a 6 hour period following the intravenous administration of ICG contained no dye. Following its intravenous injection, ICG appeared at about 8 minutes in the T-tube bile from one patient and at about 15 minutes in the other. When sampling of bile was discontinued at 6 hours after dye injection in the former case, ICG continued to be present in high concentration. Although removal from plasma was very rapid, there was distinct delay in biliary excretion, the peak concentration in bile occurring at 120 minutes (Table V). Sampling of bile was

TABLE IV

*Plasma protein-binding of indocyanine green as determined by starch block electrophoresis—average of four determinations*

| Plasma protein | Recovered ICG associated with plasma protein |
|---|---|
| | % |
| Albumin | 95.0 |
| $\alpha_1$-globulin | 2.4 |
| $\alpha_2$-globulin | 2.0 |
| $\beta$-globulin | 0.6 |
| $\gamma$-globulin | 0.0 |

TABLE V

*Appearance and disappearance of indocyanine green in bile of patient following rapid intravenous administration of indocyanine green* *

| Following dye injection | Concentration of ICG in bile |
|---|---|
| *min* | *mg/100 ml* |
| 15 | 0.010 |
| 30 | 0.022 |
| 60 | 0.283 |
| 120 | 0.750 |
| 240 | 0.354 |
| 540 | 0.163 |
| 600 | 0.134 |
| 900 | 0.067 |
| 1,020 | 0.024 |
| 1,080 | 0.005 |
| 1,140 | 0.002 |

* Two mg per kg body weight.

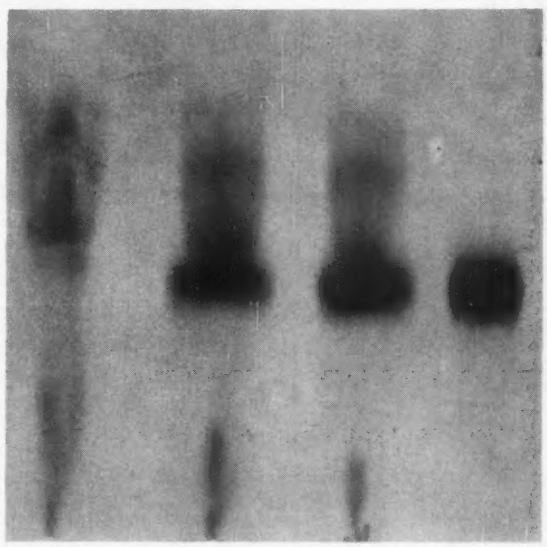


FIG. 3. ASCENDING CHROMATOGRAMS DEVELOPED IN BUFFER SYSTEM OF METHYLETHYL KETONE: PROPIONIC ACID: WATER (75: 25: 30, VOL/VOL). Left to right: human bile; human bile containing endogenously-secreted ICG; human bile containing exogenously-added ICG; ICG in aqueous solution. Green color was restricted to same regions (very dense areas) in three chromatograms containing ICG.

TABLE VI

*Peripheral arterial and venous concentrations of indocyanine green following start of intravenous dye infusion in contralateral arm*

| Following start of dye infusion | Artery concentration of ICG | Vein concentration of ICG |
|---|---|---|
| *min* | *mg/100 ml* | *mg/100 ml* |
| 5 | 0.168 | 0.166 |
| 10 | 0.164 | 0.164 |
| 15 | 0.154 | 0.156 |
| 20 | 0.147 | 0.143 |
| 25 | 0.138 | 0.142 |
| 30 | 0.128 | 0.126 |
| 35 | 0.133 | 0.137 |
| 40 | 0.136 | 0.134 |
| 45 | 0.140 | 0.139 |
| 50 | 0.152 | 0.154 |

continued in the second case until 19 hours following dye administration, at which time only a trace of ICG was present. No attempt was made at quantitative recovery of dye in the bile since T-tubes do not collect all of the biliary drainage externally. Peripheral arterial and venous samples simultaneously collected during a 50 minute period from a patient receiving an intravenous infusion of ICG revealed no consistent differences in dye concentration (Table VI). Therefore, detectable peripheral tissue uptake of ICG did not occur.

*Plasma decay.* Decay of ICG in plasma of normal subjects occurred in nearly exponential fash-

TABLE VII

*Comparison of plasma removal of indocyanine green with plasma removal of sulfobromophthalein*

| | Indocyanine green 0.5 mg/kg body weight | | | Sulfobromophthalein 5.0 mg/kg body weight | | |
|---|---|---|---|---|---|---|
| | Initial rate of decay | Half-life | Retained after 20 min | Initial rate of decay | Half-life | Retained after 45 min |
| | *%/min* | *min* | *%* | *%/min* | *min* | *%* |
| Normal | | | | | | |
| AW | 20.9 | 3.0 | 2.5 | 11.0 | 6.0 | 1.4 |
| JW | 16.2 | 3.9 | 4.5 | 15.6 | 4.1 | 1.5 |
| BO | 15.4 | 4.1 | 4.9 | 13.1 | 5.0 | 2.8 |
| FW | 15.6 | 4.2 | 4.5 | 11.1 | 6.1 | 4.4 |
| EE | 18.7 | 2.5 | 4.2 | 14.4 | 4.4 | 0.4 |
| JT | 20.1 | 3.1 | 2.2 | 12.7 | 5.1 | 1.2 |
| JM | 15.1 | 4.2 | 4.7 | 13.9 | 4.7 | 0.9 |
| CF | 20.8 | 3.0 | 3.4 | 16.0 | 4.0 | 1.3 |
| CW | 24.0 | 2.7 | 3.7 | 16.9 | 3.8 | 1.3 |
| Mean (±SD) | 18.5 (±3.1) | 3.4 (±0.7) | 3.8 (±1.0) | 13.8 (±2.1) | 4.8 (±0.8) | 1.7 (±1.2) |
| Cirrhosis—diagnosis by clinical and laboratory findings; biopsy confirmation in 7 cases | | | | | | |
| JL | 1.2 | 110.0 | 77.4 | 1.6 | 47.6 | 51.1 |
| JS | 4.6 | 14.4 | 38.2 | 2.6 | 26.4 | 33.4 |
| BB | 5.1 | 13.3 | 34.9 | 4.7 | 14.2 | 16.1 |
| AB | 1.7 | 46.8 | 70.9 | 2.8 | 29.7 | 43.1 |
| ZM | 18.8 | 3.3 | 4.2 | 9.8 | 6.8 | 5.3 |
| EC | 15.9 | 4.1 | 5.7 | 9.3 | 7.1 | 5.9 |
| JM | 6.8 | 10.0 | 24.6 | 3.8 | 19.1 | 31.6 |
| MS | 4.0 | 44.8 | 72.5 | 2.5 | 27.5 | 33.0 |
| DG | 3.3 | 20.4 | 50.6 | 2.1 | 43.0 | 49.0 |
| JC | 2.9 | 23.6 | 55.4 | 1.8 | 47.0 | 50.7 |
| MM | 14.6 | 4.4 | 7.7 | 5.1 | 13.0 | 17.9 |
| AV | 15.0 | 4.3 | 5.7 | 5.7 | 11.9 | 18.0 |
| Mean | 7.8 | 25.0 | 37.3 | 4.3 | 24.4 | 29.6 |
| Cirrhosis—diagnosis by biopsy; all clinical and laboratory findings normal | | | | | | |
| BM | 17.0 | 3.7 | 5.0 | 12.2 | 5.6 | 6.2 |
| RR | 16.4 | 3.9 | 4.4 | 11.2 | 5.8 | 1.2 |
| Viral hepatitis | | | | | | |
| RB | 16.0 | 3.9 | 5.1 | 6.3 | 21.4 | 23.4 |
| EG | | | | | | |
| 5/1/59 | 16.5 | 3.9 | 5.3 | 4.0 | 20.2 | 34.7 |
| 5/8/59 | 20.0 | 3.1 | 2.5 | 9.9 | 6.8 | 7.3 |
| 5/18/59 | 19.3 | 3.1 | 2.8 | 10.3 | 6.2 | 3.7 |

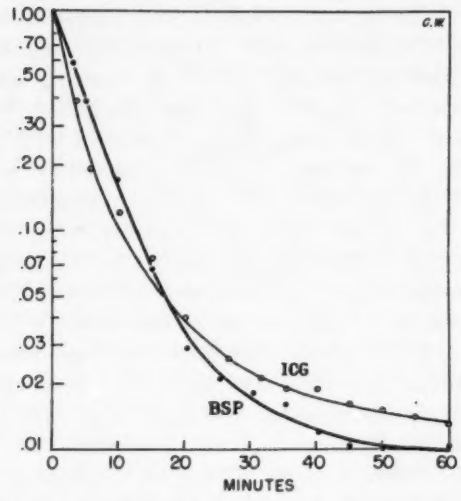



FIG. 4. DECAY OF ICG AND BSP IN PLASMA OF CONTROL SUBJECT (CW), PLOTTED WITH ZERO-TIME CONCENTRATION TAKEN AS UNITY.

ion for 10 to 20 minutes, exhibiting falling concentrations on a straight line on semilogarithmic paper. Following that initial period, deceleration of decay occurred in all cases (Figure 4). The approximate rate of decay, calculated during the exponential decay segment, was 18.5 per cent per minute (SD 3.1) for ICG as compared with 13.8 per cent per minute (SD 2.1) for BSP (Table VII).

Among the control subjects of this series, the mean BSP level 45 minutes after injection was 1.7 per cent (SD 1.2) of the initial concentration. ICG removal was so rapid that the percentage retained after 20 minutes was selected for comparison. The mean value in the normal group was 3.85 per cent (SD 1.0). When the amount of ICG injected was raised to 1 mg per kg body weight, the percentage retained at 20 minutes remained unchanged (mean, 3.9 per cent). When the dosage was further increased to 2.0 mg per kg body weight, the mean value for 20 minute retention was 4.0 per cent.

Plasma decay of ICG characteristically was suppressed in patients with cirrhosis. An example of marked defect in dye clearance is illustrated in Figure 5. The BSP decay curve is shown on the same plot for comparison. The group of subjects with cirrhosis contained patients with widely differing degrees of liver damage (Table II). Rates of ICG decay in plasma of those patients, as expected, varied markedly as did decay rates for BSP (Table VII). Average values for removal of both dyes, therefore, have little meaning in that group. The correlation "R" between initial rates of decay for the two test substances was 0.92 ($p < 0.01$). Two patients with cirrhosis had no clinical stigmata of that disease and showed no evidence of impaired liver function. They nevertheless had histological liver changes which unequivocally established the diagnosis of cirrhosis. If those two are excluded, the correlation is 0.91 ($p < 0.01$). Although the mean value for initial decay rate of ICG has a high degree of correlation with the mean initial decay rate of BSP within the group of patients with cirrhosis, there are individual cases that belie the average values (Table VII). In most cases ICG was removed somewhat more rapidly than BSP. Two patients (MM and AV) with distinctly abnormal BSP decay curves cleared ICG in normal fashion. Two patients (AB and JL) with extreme impairment of hepatic function removed BSP more rapidly than ICG.

Both patients with infectious hepatitis had distinctly abnormal BSP decay curves but probably normal rates of removal and 20 minute levels of ICG (Table VII). One of those patients (EG) had two repeat studies during convalescence. BSP removal showed a return to normal, while ICG removal, already within the normal range, nevertheless showed definite increase in rate of plasma disappearance.

During the course of plasma decay studies, ICG

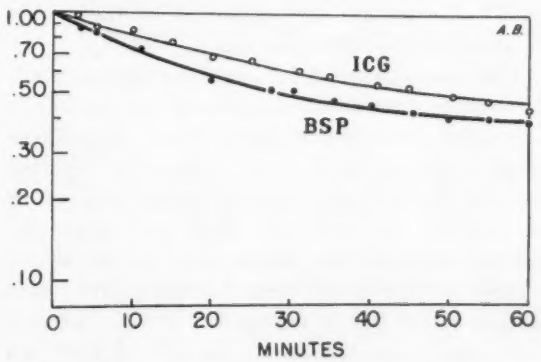



FIG. 5. DECAY OF ICG AND BSP IN PLASMA OF PATIENT WITH DECOMPENSATED CIRRHOSIS (AB), PLOTTED WITH ZERO-TIME CONCENTRATION TAKEN AS UNITY.

was inadvertently extravasated subcutaneously in three subjects. No ill effects resulted. Neither single nor repeated injections or infusions of ICG were associated with untoward systemic reactions.

*Hepatic extraction.* Stabilization of both arterial and hepatic venous levels of ICG usually occurred within 20 minutes and could be maintained for periods as long as 2 hours. Extraction rates of ICG determined at the earliest point of plasma dye stabilization varied between 0.59 and 0.83 in seven normal subjects. Estimated hepatic blood flow calculated from these data ranged from 0.70 to 1.80 L per minute per $m^2$ of body surface area. The results of studies of hepatic flow will be reported in detail in a later communication (8).

## DISCUSSION

Indocyanine green appears to be rapidly and completely bound to plasma protein following its intravenous administration. This can be inferred from the data which show that at zero time the space occupied by the dye is about the same as that of plasma volume (Table I). If it were not bound to a nondiffusible molecule, an appreciable amount of the dye would quickly leave the intravascular compartment. The shift which occurs in the absorption spectrum of ICG when albumin is present in solution (Figure 2) is evidence that the dye is bound to albumin. The similarity of the absorption spectrum of the dye in human plasma and in albumin solution, and the identity of the absorption maxima suggest that plasma proteins other than albumin are not important in the binding of ICG. These data are supported by the results of starch block electrophoresis (Table II).

Chromatography of ICG in three different buffer systems fails to show evidence that the dye is secreted into bile in conjugated form. ICG differs in that respect from BSP which has been reported to be secreted, in part, in conjugation with one or more amino acids (9–12). The present studies further indicate that, unlike BSP, ICG is not cleared by the kidney and that peripheral tissue uptake of the dye is negligible. Renal clearance (13) and tissue uptake (6, 14, 15) of BSP are known to occur.

In normals and in most patients with cirrhosis ICG is removed more rapidly than is BSP. It appears likely, on the basis of decay characteristics of ICG in patients with cirrhosis, that the dye has good discriminative value in selecting those subjects who have a significant degree of liver disease. In the entire group of patients with cirrhosis, its sensitivity compared favorably with BSP, the correlation "R" between the initial decay rate of the two test substances being 0.92 ($p < 0.01$). ICG appears in a less favorable light when individual cases are examined, however, for it revealed no abnormality in two patients (AV and MM) in whom BSP decay was significantly impaired. At the same time, ICG was more strikingly abnormal in two jaundiced cirrhotics (JL and AB) than was BSP. In the two cases of viral hepatitis studied, ICG failed entirely to reflect hepatic dysfunction. At the time the dye studies were made the clinical disease was mild, and both patients had normal serum bilirubin concentrations and cephalin flocculation tests.

There are several possible explanations for the failure, in certain subjects, of the two dyes to behave similarly. First, it is known that the mechanisms whereby the two substances are handled by the liver are not identical; BSP is partially conjugated during its biliary secretion while ICG is not. There may be other differences, not recognized at present, between clearance mechanisms of the two dyes. On the other hand it may be that physiologically equivalent amounts of BSP and ICG had not been given. The dosage of 0.5 mg per kg body weight was most extensively used in these studies. On the basis of seven ICG decay curves when 1.0 mg per kg body weight had been injected, there is no indication that dye decay is different at these two dosage levels. The percentage of initial plasma dye concentration remaining at 20 minutes was the same in both cases. A dose of 2.0 mg per kg was studied in three subjects. The percentage of dye remaining at 20 minutes and the shape of the decay curve did not differ significantly from those found when ICG had been given in the two smaller amounts. Nothing is known of the toxicity of ICG in amounts exceeding 2.0 mg per kg body weight. Investigations using such large amounts of dye were therefore not undertaken.

Another explanation for the occasional disparate behavior of the two dyes lies in the fact that BSP plasma decay is partially accounted for by extra-

hepatic removal, while no such extraction has been demonstrated for ICG. In hepatectomized, eviscerated dogs, 50 per cent of the initially-injected dose of BSP has been shown to be removed in 50 minutes (13). It is likely, therefore, that in subjects with very severe liver disease extrahepatic modes of BSP removal might become very significant. In these patients ICG may be a better relative index of liver function because there is apparently no alternate means for removal.

For clinical purposes, the retained percentage of ICG, 20 minutes after a single injection of 0.5 mg per kg body weight, seems to give information equivalent to the rate of decay during the exponential portion of the plasma decay curve. In this small series, inadequate to establish norms, it appeared that 20 minute dye retention of less than 5 to 6 per cent and initial decay of greater than 15 per cent per minute were within normal limits.

If its insensitivity to minor degrees of liver dysfunction limits its practical value in the evaluation of liver disease, ICG seems to be an excellent substance for estimating hepatic blood flow by the Fick principle (8). It is apparently extracted from the plasma exclusively by the liver. The faster decay of ICG than of BSP in some subjects with mild liver disease may mean that blood flow studies can be carried out in such persons with ICG when those studies would not be feasible with BSP because of poor hepatic extraction and excessive plasma retention of the dye. ICG extraction rates of a magnitude suitable for calculating hepatic blood flow were found in these studies.

## SUMMARY AND CONCLUSIONS

Following its intravenous injection, indocyanine green (ICG) appears to be rapidly and completely bound to plasma protein, of which albumin is the principal carrier. The dye is excreted in bile in unconjugated form. It is not cleared by extrahepatic mechanisms in detectable amounts. ICG was nonirritating when inadvertently introduced subcutaneously and produced no untoward reactions upon single or repeated intravenous injections or infusions.

In normal subjects and in selected patients with liver disease, plasma decay of ICG was similar to that of sulfobromophthalein (BSP). In subjects without liver disease mean initial ICG decay rate was 18.5 per cent per minute as compared with 13.8 per cent for BSP. In patients with cirrhosis, the correlation "R" between initial decay rates of the two substances was 0.92. While patients with mild liver disease seem to remove ICG more rapidly than BSP, persons with marked hepatic dysfunction may remove BSP more rapidly than ICG.

Indocyanine green should provide a reliable means of estimating hepatic blood flow by use of the Fick principle. Its physical properties, degree of hepatic removal, tolerance by human subjects, and rapid decay even in the presence of mild liver disease make it an ideal dye for that purpose.

## ACKNOWLEDGMENTS

The authors gratefully acknowledge the advice and assistance of Dr. Walter H. Abelmann, Dr. Rudi Schmid and Dr. Charles S. Lieber, and the technical assistance of the Misses Lois M. Betten, Lydia E. Hammaker, Felicitas Honegger, and Mary T. Collins.

We are indebted to Hynson, Westcott, and Dunning, Inc., of Baltimore, Md., for giving us very generous supplies of Cardio-Green.

## REFERENCES

1. Fox, I. J., Brooker, L. G. S., Heseltine, D. W., Essex, H. E., and Wood, E. H. A tricarbocyanine dye for continuous recording of dilution curves in whole blood independent of variations in blood oxygen saturation. Proc. Mayo Clin. 1957, **32**, 478.
2. Fox, I. J., and Wood, E. H. Applications of dilution curves recorded from the right side of the heart or venous circulation with the aid of a new indicator dye. Proc. Mayo Clin. 1957, **32**, 541.
3. Wheeler, H. O., Cranston, W. I., and Meltzer, J. I. Hepatic uptake and biliary excretion of indocyanine green in the dog. Proc. Soc. exp. Biol. (N. Y.) 1958, **99**, 11.
4. Leevy, C. M., Stein, S. W., Cherrick, G. R., and Davidson, C. S. Indocyanine green clearance: A test of liver excretory function. Clin. Res. 1959, **7**, 290.
5. Rapaport, E., Ketterer, S. G., and Wiegand, B. D. Hepatic clearance of indocyanine green. Clin. Res. 1959, **7**, 289.
6. Ingelfinger, F. J., Bradley, S. E., Mendeloff, A. I., and Kramer, P. Studies with bromsulphalein: Its disappearance from the blood after a single intravenous injection. Gastroenterology 1948, **11**, 646.
7. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J. The estimation of hepatic blood flow in man. J. clin. Invest. 1945, **24**, 890.

8. Stein, S. W., Abelmann, W. H., Leevy, C. M., Cherrick, G. R., and Lieber, C. S. Hepatic blood flow of man estimated by hepatic clearance of indocyanine green, with observations on the effect of intravenous alcohol. In preparation.
9. Grodsky, G. M., Carbone, J. V., and Fanska, R. Metabolism of sulphobromophthalein. Nature (Lond.) 1959, **183**, 469.
10. Combes, B. Biliary excretion by the rat of bromsulfalein as a conjugate of glycine and glutamic acid. Science 1959, **129**, 388.
11. Meltzer, J. I., Wheeler, H. O., and Cranston, W. I. Metabolism of sulfobromophthalein sodium (BSP) in dog and man. Proc. Soc. exp. Biol. (N. Y.) 1959, **100**, 174.
12. Carbone, J. V., Grodsky, G. M., and Fanska, R. Chemical and clinical studies of bromsulfalein (BSP) metabolites. J. clin. Invest. 1959, **38**, 994.
13. Cohn, C., Levine, R., and Streicher, D. The rate of removal of intravenously injected bromsulphalein by the liver and extrahepatic tissues of the dog. Amer. J. Physiol. 1947, **150**, 229.
14. Combes, B., Wheeler, H. O., Childs, A. W., and Bradley, S. E. The mechanisms of bromsulfalein removal from the blood. Trans. Ass. Amer. Phycns 1956, **69**, 276.
15. Brauer, R. W., Pessotti, R. L., and Krebs, J. S. The distribution and excretion of $S^{35}$-labeled sulfobromophthalein-sodium administered to dogs by continuous infusion. J. clin. Invest. 1955, **34**, 35.

# STUDIES ON THE MECHANISM OF THE PLASMA 17–HYDROXY–CORTICOSTEROID ELEVATION INDUCED IN MAN BY ESTROGENS *

By ELEANOR Z. WALLACE AND ANNE C. CARTER

*(From the Department of Medicine, State University of New York, College of Medicine at New York City, and the Medical Service (Division II), Kings County Hospital, Brooklyn, N. Y.)*

(Submitted for publication September 15, 1959; accepted December 4, 1959)

The administration of estrogens to man produces sustained elevation above normal levels of plasma 17-hydroxycorticosteroids (17-OHCS) (1, 2). The elevated levels of plasma 17-OHCS are associated with an augmented response of plasma levels of these steroids to exogenous adrenocorticotropic hormone (ACTH) and with a marked delay in the rate of clearance of exogenous cortisol from plasma (2). The last trimester of pregnancy is associated with similar changes in levels of plasma 17-OHCS and in their response to exogenous ACTH (3–9). In the last trimester of pregnancy there is a variable increase in urinary 17-OHCS excretion, a decrease in the rate of clearance of exogenous cortisol from plasma (9–11), a decrease in the rate of clearance of exogenous tetrahydrocortisone from plasma (11) and an increase in corticosteroid-binding protein (12). The apparently normal adrenal status of pregnant women and of patients treated with estrogens for long periods of time suggests that the administration of estrogens to man elevates plasma 17-OHCS levels by altering the normal metabolism of endogenous cortisol by a mechanism similar to that seen in pregnancy. Studies have been made of urinary 17-OHCS excretion, of clearance of tetrahydrocortisone from plasma, and of plasma corticosteroid-binding protein after the administration of estrogens to man.

## MATERIALS AND METHODS

The patients studied were primarily adult females ranging in age from 37 to 75 years, all of whom either had been surgically castrated or were at least 3 years past a spontaneous menopause. A number of these subjects were patients with metastatic carcinoma of the breast. One male was studied during recovery from a myocardial infarction.

After appropriate control studies, the patients were treated for periods of 2 weeks to 6 months with either oral ethinyl estradiol (Estinyl), diethylstilbestrol or conjugated equine estrogens (Premarin). Only doses of these preparations which uniformly elevated plasma 17-OHCS levels above normal were used in the studies (Table I). Ethinyl estradiol 0.1 and 0.5 mg, diethylstilbestrol 15 mg, and Premarin 10 mg, daily by mouth, all caused significant increases in the levels of plasma 17-OHCS.

The effect of oral ethinyl estradiol, 0.5 mg daily, on urinary excretion of free and total 17-OHCS and of 17-ketosteroids was studied. Twenty-four hour urinary excretion of steroids was determined in each patient before estrogen therapy and again after plasma 17-OHCS levels had become elevated. All determinations were performed on 24-hour urines collected under refrigeration. Urinary creatinine determinations were made on all specimens by the method of Bonsnes and Taussky (13). Urinary 17-ketosteroids were measured by the Gibson and Norymberski modification (14) of the method of Drekter and associates. Urinary 17-OHCS were determined before (free 17-hydroxycorticosteroids) and after (total 17-hydroxycorticosteroids) hydrolysis with β-glucuronidase,[1] by the modification of the Silber-Porter technique of Peterson and colleagues (15).

TABLE I

*Effect of various estrogen preparations on plasma 17-hydroxycorticosteroids*

| Therapy | Dose p.o. | Subjects | Plasma 17-OHCS | |
|---|---|---|---|---|
| | | | Control | After therapy |
| | *mg/day* | *no.* | *μg/100 ml* | |
| Ethinyl estradiol | 0.1 | 5 | 23 ± 5.0* | 46 ± 12.2 |
| Ethinyl estradiol | 0.5 | 18 | 15 ± 6.0 | 58 ± 18.1 |
| Stilbestrol | 15.0 | 8 | 19 ± 4.1 | 54 ± 10.1 |
| Conjugated estrogens (Premarin) | 10.0 | 5 | 24 ± 4.8 | 42 ± 5.0 |

* Mean ± standard deviation.

* This work was supported in part by a research grant from The National Cancer Institute, Bethesda, Md.

[1] Ketodase, Warner-Chilcott.

The clearance from plasma of exogenous tetrahydrocortisone was studied in 7 patients in whom plasma 17-OHCS levels had been elevated by estrogen therapy and in 4 control subjects. Seventy-five mg of crystalline tetrahydrocortisone was dissolved in 7 to 8 ml of hot alcohol, added to 80 to 100 ml of normal saline and administered intravenously over a period of 2 to 5 minutes. Plasma samples were obtained prior to and at approximately 20, 40, 60 and 90 minutes after each infusion. Plasma 17-OHCS were determined by the method of Peterson and colleagues (15).

The binding of cortisol to a corticosteroid-binding globulin, transcortin, was kindly determined by Drs. Sandberg and Slaunwhite by the method which they have described (12, 16). Binding was determined by dialyzing 10 ml of diluted plasma (1:5 with physiological saline) against 30 ml of saline, containing approximately 0.3 $\mu$g of $C^{14}$ cortisol at 4° C. Binding was also determined by the addition of 1 $\mu$g of cortisol to the saline in order to measure the decrease in the binding caused by the addition of cortisol—"transcortin capacity." The binding capacity is inversely related to the decrease in binding caused by the addition of 1 $\mu$g of cortisol. Plasma 17-OHCS levels, transcortin-binding and transcortin capacity were measured in 12 patients, 6 of whom previously have been reported (16), before and at 2 to 3 day intervals after the institution of therapy.

## RESULTS

*1. Effect of oral ethinyl estradiol (0.5 mg per day) upon urinary excretion of steroids.* Ethinyl estradiol administration for periods of 2 to 4 weeks resulted in a significant decrease ($p < 0.01$) in the mean excretion of total urinary 17-hydroxycorticosteroids in nine patients studied (Table II). Since the excretion of free urinary 17-OHCS was not significantly altered by the administration of estrogens, the major decrease in urinary 17-OHCS appeared to be in the conjugated fraction.

TABLE II

*Urinary steroid excretion before and after ethinyl estradiol* *

| | Patients | Control excretion | Postestrogen excretion | p† |
|---|---|---|---|---|
| | *no.* | *mg/24 hrs* | *mg/24 hrs* | |
| Total urinary 17-OHCS | 9 | 4.3 ± 1.4‡ | 3.1 ± 1.5 | <0.01 |
| Free urinary 17-OHCS | 8 | 0.51 ± 0.19 | 0.67 ± 0.46 | >0.05 |
| Urinary 17-KS | 6 | 8.2 ± 3.5 | 7.7 ± 2.3 | >0.05 |

* *Per os*, 0.5 mg per day.
† Determined by *t* test.
‡ Mean ± standard deviation.

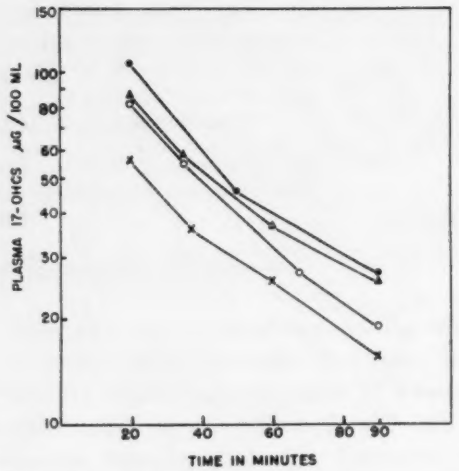



FIG. 1. SEMILOGARITHMIC GRAPH OF RATE OF FALL OF PLASMA 17-HYDROXYCORTICOSTEROIDS AFTER RAPID INTRAVENOUS INJECTION OF TETRAHYDROCORTISONE, 75 MG, IN FOUR NORMAL SUBJECTS.

Mean urinary 17-ketosteroid excretion was unaltered by estrogen therapy ($p > 0.05$).

*2. Effect of the administration of estrogens upon the clearance of exogenous tetrahydrocortisone from plasma.* The rate of disappearance of intravenously administered tetrahydrocortisone from plasma of four control subjects and of seven patients in whom plasma 17-OHCS levels had been elevated by the administration of either diethylstilbestrol or ethinyl estradiol is shown in Figures 1 and 2. The mean decline for each group is shown in Figure 3. The group treated with estrogens cleared intravenously administered tetrahydrocortisone from blood at a significantly ($p < 0.01$) slower rate than did the control group. The decline per minute of plasma 17-OHCS levels was 1.86 and 0.69 per cent in the control and treated groups, respectively.

*3. The effect of administration of estrogens upon the binding of cortisol to transcortin.* The effects of ethinyl estradiol, diethylstilbestrol and Premarin on transcortin-binding of cortisol were identical (Table III). Therapy with these hormones produced an increase in the percentage of cortisol bound to transcortin from mean levels of 90 ± 4.1 per cent cortisol bound before therapy to 97 ± 1.4 per cent bound after therapy when levels of plasma 17-OHCS had become elevated ($p < 0.01$). In addition, estrogen therapy pro-

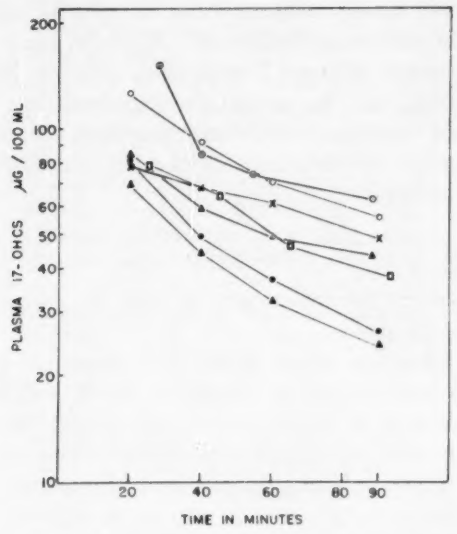



FIG. 2. SEMILOGARITHMIC GRAPH OF RATE OF FALL OF PLASMA 17-HYDROXYCORTICOSTEROIDS AFTER RAPID INTRAVENOUS INJECTION OF TETRAHYDROCORTISONE, 75 MG, IN SEVEN ESTROGEN-TREATED SUBJECTS.

duced a significant increase in transcortin-binding capacity when this was determined by the suppression of binding following the addition of 1 µg of cortisol (F) to the binding system. There was a mean decrease of $23 \pm 3.9$ per cent bound in the control subjects and of $12 \pm 2.6$ per cent bound in the patients treated with estrogens. The increase in transcortin-binding capacity was significant at the 1 per cent level as determined by the *t* test.

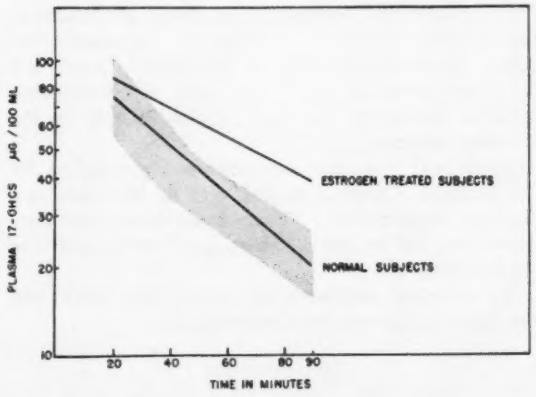



FIG. 3. COMPARISON OF TETRAHYDROCORTISONE CLEARANCE RATES IN CONTROL AND ESTROGEN-TREATED SUBJECTS. Solid lines indicate calculated means for the four control and seven estrogen-treated subjects. Stippled area indicates the extreme limits of variation for the control group. The mean percentage decreases of plasma 17-hydroxycorticosteroid levels per minute for estrogen-treated subjects is significantly less than that for control subjects at the 1 per cent level.

TABLE III

*Effect of estrogen therapy on binding of cortisol (F) to transcortin in 12 patients*

| | Before therapy | During therapy |
|---|---|---|
| Plasma 17-OHCS (µg/100 ml) | $20 \pm 5.7$* | $50 \pm 11.8$ |
| Cortisol binding before 1 µg F (% bound) | $90 \pm 4.1$ | $97 \pm 1.4$ |
| Decrease in % bound after 1 µg F | $23 \pm 3.9$ | $12 \pm 2.6$ |

* Mean ± standard deviation.

## DISCUSSION

There has been increased interest in alterations in the metabolism of 17-hydroxycorticosteroids (17-OHCS) induced by pregnancy and by the administration of estrogens in man. The demonstration that orally administered diethylstilbestrol produced elevations of plasma 17-OHCS levels (1) in man has led to a number of studies designed to define the mechanism of these increases.

The description by Daughaday (17–19) of a specific corticosteroid-binding globulin in human plasma has stimulated investigations of the effects of pregnancy and of estrogen administration upon the protein-binding of plasma 17-OHCS. Slaunwhite and Sandberg (12, 16), using different conditions of dialysis, have reported increases in a corticosteroid-binding protein (transcortin) during the administration of diethylstilbestrol and ethinyl estradiol. In the studies reported here, increases in the percentage of cortisol bound to transcortin and in transcortin-binding capacity are shown to occur with the administration of Premarin as well as with diethylstilbestrol and ethinyl estradiol. Evidence that the elevated Porter-Silber chromogens present after estrogen therapy are cortisol has been reported by Peterson on the basis of isotope dilution and isotope derivative assays of the plasma cortisol (20).

If cortisol bound to corticosteroid-binding protein is physiologically "inactive" and less available for metabolic disposal than is unbound corti-

sol (12), then many of the changes in 17-OHCS metabolism induced by estrogens may be explained by increased binding of plasma cortisol to a specific globulin. Increased levels of plasma 17-OHCS, without clinical evidence of hyper- or hypoadrenocorticism, imply the presence of increased levels of protein-bound cortisol and normal amounts of cortisol not bound to the specific binding protein. The absence of adrenal suppression, as manifested by augmented response of levels of plasma 17-OHCS to ACTH, further suggests that the "active" moiety of circulating cortisol is not present in excess. These findings suggest that the circulating increased levels of plasma 17-OHCS do not suppress the pituitary or adrenal glands.

The quantitatively exaggerated rise in levels of 17-OHCS after corticotropin may be explained, at least in part, by the delay in clearance of cortisol from plasma. The delayed plasma clearance of cortisol is presumably due to the decreased availability of the transcortin-bound fraction of cortisol for metabolism and conjugation by the liver. The decrease in conjugated urinary 17-OHCS may also be explained by such a sequence of events. The unchanged levels of free cortisol in the urine found in this study are compatible with the clinically normal adrenal state of patients receiving estrogens.

The delayed clearance of exogenous tetrahydrocortisone, a steroid not bound in any significant degree by corticosteroid-binding globulin (18), cannot be explained by increases in the binding capacity of this protein. The existence of a protein-binding system for tetrahydrocortisone, as yet undescribed, which may be influenced by the administration of estrogens, should be considered. There is evidence for a diversity of protein-binding systems affected by estrogens in the reported increases in thyroid-binding globulin induced by the administration of diethylstilbestrol (21).

The similarity in changes produced by adequate doses of estrogens and those that occur spontaneously in pregnancy is striking. All of the alterations in steroid metabolism induced by administration of estrogens, except for the decrease in conjugated urinary corticosteroids, occur in the last trimester of pregnancy. The exact physiological significance of the altered corticosteroid metabolism during pregnancy and estrogen administration remains to be defined. Although many of the changes in steroid metabolism noted in both situations can be adequately explained by increases in corticosteroid-binding protein, the data do not exclude the possibility of other contributing mechanisms.

## SUMMARY

Estrogen administration to man in adequate dosage produces:

1. Elevation above normal of plasma 17-hydroxycorticosteroid (17-OHCS) levels and excessive rise of these steroids after intravenous ACTH without clinical hyperadrenocorticism.
2. Decreased urinary excretion of conjugated 17-OHCS.
3. Delay in rate of clearance from plasma of exogenous cortisol and tetrahydrocortisone.
4. Increase in cortisol-binding to corticosteroid-binding proteins (transcortin) and in transcortin-binding capacity. This mechanism can explain the reported findings, excepting the diminished rate of clearance from plasma of exogenous tetrahydrocortisone which remains unexplained by the data reported.

## ACKNOWLEDGMENTS

The authors are indebted to Dr. Avery A. Sandberg, Roswell Park Memorial Institute, for the transcortin-binding determinations and to Mr. Robert Israel and Dr. John Fertig of the Department of Biostatistics, Columbia University, for their invaluable help in the statistical analyses.

Estinyl and Premarin were generously supplied by The Schering Corporation, and Ayerst, McKenna and Harrison, respectively. Tetrahydrocortisone was generously supplied by Merck, Sharp and Dohme; and The Upjohn Company.

The technical assistance of James McCarrick and Joel Spivak is gratefully acknowledged.

## REFERENCES

1. Taliaferro, I., Cobey, F., and Leone, L. Effect of diethylstilbestrol on plasma 17-hydroxycorticosteroid levels in humans. Proc. Soc. exp. Biol. (N. Y.) 1956, **92**, 742.
2. Wallace, E. Z., Silverberg, H. I., and Carter, A. C. Effect of ethinyl estradiol on plasma 17-hydroxycorticosteroids, ACTH responsiveness and hydrocortisone clearance in man. Proc. Soc. exp. Biol. (N. Y.) 1957, **95**, 805.

3. Gemzell, C. A. Blood levels of 17-hydroxycorticosteroids in normal pregnancy. J. clin. Endocr. 1953, **13**, 898.
4. Morris, C. J. O. R., and Williams, D. C. Estimation of individual adrenocortical hormones in human peripheral blood. Ciba Found. Coll. Endocr. 1953, **7**, 261.
5. Robinson, H. J., Bernhard, W. G., Grubin, H., Wanner, H., Sewekow, G. W., and Silber, R. H. 17,21-Dihydroxy-20-ketosteroids in plasma during and after pregnancy. J. clin. Endocr. 1955, **15**, 317.
6. Wallace, E. Z., Christy, N. P., and Jailer, J. W. Clinical application of the simplified Silber-Porter method for determining plasma 17-hydroxycorticosteroids. J. clin. Endocr. 1955, **15**, 1073.
7. Bayliss, R. I., Browne, J. C., Round, B. P., and Steinbeck, A. W. Plasma 17-hydroxycorticosteroids in pregnancy. Lancet 1955, **1**, 62.
8. Assali, N. S., Garst, J. B., and Voskian, J. Blood levels of 17-hydroxycorticosteroids in normal and toxemic pregnancies. J. Lab. clin. Med. 1955, **46**, 385.
9. Cohen, M., Stiefel, M., Reddy, W. J., and Laidlaw, J. C. The secretion and disposition of cortisol during pregnancy. J. clin. Endocr. 1958, **38**, 1076.
10. Migeon, C. J., Bertrand, J., and Wall, P. E. Physiological disposition of 4-$C^{14}$-cortisol during late pregnancy. J. clin. Invest. 1957, **36**, 1350.
11. Christy, N. P., Wallace, E. Z., Gordon, W. E. L., and Jailer, J. W. On the rate of hydrocortisone clearance from plasma in pregnant women and in patients with Laennec's cirrhosis. J. clin. Invest. 1959, **38**, 299.
12. Slaunwhite, W. R., Jr., and Sandberg, A. A. Transcortin: A corticosteroid-binding protein of plasma. J. clin. Invest. 1959, **38**, 384.
13. Bonsnes, R. W., and Taussky, H. H. On the colorimetric determination of creatinine by the Jaffé reaction. J. biol. Chem. 1945, **158**, 581.
14. Gibson, G., and Norymberski, J. K. A note on the rapid assay of 17-ketogenic steroids in urine. Ann. rheum. Dis. 1954, **13**, 59.
15. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bunim, J. J. The physiological disposition and metabolic fate of hydrocortisone in man. J. clin. Invest. 1955, **34**, 1779.
16. Sandberg, A. A., and Slaunwhite, W. R., Jr. Transcortin: A corticosteroid-binding protein of plasma. II. Levels in various conditions and the effects of estrogens. J. clin. Invest. 1959, **38**, 1290.
17. Daughaday, W. H. Binding of corticosteroids by plasma proteins. I. Dialysis equilibrium and renal clearance studies. J. clin. Invest. 1956, **35**, 1428.
18. Daughaday, W. H. Binding of corticosteroids by plasma proteins. III. The binding of corticosteroid and related hormones by human plasma and plasma protein fractions as measured by equilibrium dialysis. J. clin. Invest. 1958, **37**, 511.
19. Daughaday, W. H. Binding of corticosteroids by plasma proteins. IV. The electrophoretic demonstration of corticosteroid binding globulin. J. clin. Invest. 1958, **37**, 519.
20. Peterson, R. E. The miscible pool and turnover rate of adreno-cortical steroids in man. Recent Progr. Hormone Res. 1959, **15**, 231.
21. Dowling, J. T., Freinkel, N., and Ingbar, S. H. Effect of diethylstilbestrol on the binding of thyroxine in serum. J. clin. Endocr. 1956, **16**, 1491.

# FREE FATTY ACID MOBILIZATION BY NEUROADRENERGIC STIMULATION IN MAN *

By JAMES T. HAMLIN, III,† ROGER B. HICKLER AND ROBERT G. HOSKINS ‡

*(From the Medical Service and the Pearl Geriatric Unit of the Peter Bent Brigham Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass.)*

(Submitted for publication September 16, 1959; accepted December 4, 1959)

A role of the sympathetic nervous system in the metabolism of adipose tissue has been suggested by clinical observations and by animal denervation experiments (1). The recent observations of the mobilization of free fatty acids[1] (FFA) by norepinephrine (NE) *in vitro* (2) and *in vivo* (3, 4) add presumptive evidence for a function of the sympathetic nervous system in the transport of fatty acids. To elucidate further the role of the sympathetic nervous system in fatty acid transport, the effect of a standard physiological stress on plasma FFA concentration was studied.

Previous work has shown that the normal individual defends against the orthostatic stress of a 60° upright tilt by an increase in diastolic blood pressure and pulse rate. This response is probably mediated by the autonomic nervous system through baroceptor reflexes and is accompanied by a rise in plasma NE concentration (5). These responses reflect an increase in neuroadrenergic activity; and, as a corollary, are absent in individuals after sympathectomy and in patients with primary autonomic insufficiency.

It is the purpose of this report to present the effects of neuroadrenergic stimulation produced by orthostatic stress on plasma FFA concentration.

## METHODS

The response of plasma FFA and NE to tilting was studied in 6 normal subjects, in 1 patient with bilateral adrenalectomy, and in 1 patient with primary autonomic insufficiency. All studies were carried out with fasting subjects in the supine position on a tilt table. An indwelling needle was secured in an antecubital vein and was kept patent for the collection of blood specimens by a slow infusion of sterile isotonic saline. Pulse rate and blood pressure were recorded until a steady state was achieved. The subject was then tilted 60° upright for 30 minutes. Pulse and blood pressure were recorded at regular intervals throughout the experiment. Blood specimens for plasma FFA and NE were drawn 10 minutes before and immediately prior to tilting to 60° upright and at 2.5, 5, 10, 20 and 30 minutes in the upright position. The subject was then returned to the supine position and another blood specimen was drawn 10 minutes later.

The effect of an adrenolytic agent, phentolamine methanesulfonate,[2] on the plasma FFA response to tilting was studied in 3 normal individuals. Each subject served as his own control. The FFA and NE responses were determined during a 30 minute control tilt after which 5 mg of phentolamine was administered intravenously and the subject was tilted a second time. One subject was studied again 1 month later with a dose of 10 mg of phentolamine.

The effect of 2 successive tilts was studied in 3 normal subjects without administering phentolamine prior to the second tilt.

All blood specimens were collected in tubes with phenol-free heparin as the anticoagulant. Specimens were kept in ice and at the end of the experiment were centrifuged and the plasma stored at −18° C for subsequent FFA and NE determinations. FFA concentrations were determined by the method of Dole (6) and the NE concentrations by the method of Weil-Malherbe and Bone (7) as modified by Aronow and Howard (8).

## RESULTS

The response of plasma FFA concentration to tilting in six normal individuals is illustrated in Figures 1A and 1B and in Table I. In each case there was a rise above control levels in 2.5 minutes and a peak increment in 20 to 30 minutes. The FFA concentration of the specimen collected immediately prior to tilting was used as the con-

* Supported in part by grants from the John A. Hartford Foundation, Inc., New York, N. Y.

† Public Health Service Postdoctoral Fellow, National Institute of Mental Health, Bethesda, Md. Present address: New York Medical College, Flower and Fifth Avenue Hospitals, New York, N. Y.

‡ Public Health Service Postdoctoral Fellow, National Heart Institute, Bethesda, Md.

[1] The term "free fatty acids" is used here in place of the terms "nonesterified fatty acids" and "unesterified fatty acids" previously used to describe this lipid fraction.

[2] Kindly supplied by the Ciba Pharmaceutical Co. as Regitine.

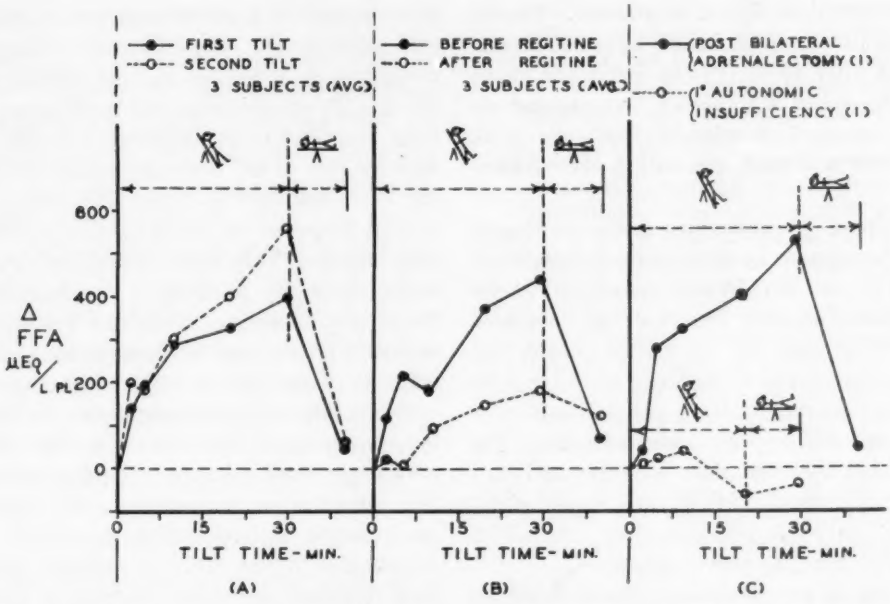



FIG. 1. RELATIONSHIP BETWEEN ORTHOSTATIC STRESS AND PLASMA FFA CONCENTRATION. (A) Effect of successive tilting; (B) effect of Regitine; (C) effect of bilateral adrenalectomy and primary autonomic insufficiency.

trol level. Maximal increments above control values varied from 250 to 870 μEq per L with a mean increment of 480 μEq per L, an increase of approximately 70 per cent in plasma FFA concentration. After returning the subject to 0°, the FFA concentration fell rapidly and approached

TABLE I

*Free fatty acid and norepinephrine response to 30 minute tilt* *

| Subject | Control | | 2.5 min | | 5 min | | 10 min | | 20 min | | 30 min | | 40 min | | Remarks |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | FFA | NE | FFA | NE | FFA | NE | FFA | NE | FFA | NE | FFA | NE | FFA | NE | |
| | μEq/L | μg/L | μEq/L | μg/L | μEq/L | μg/L | μEq/L | μg/L | μEq/L | μg/L | μEq/L | μg/L | μEq/L | μg/L | |
| RBH (1) | 435 | 3.1 | 580 | 3.1 | 690 | 3.1 | 640 | 4.0 | 865 | 2.6 | 1,310 | 3.2 | 680 | 4.1 | Normal subject |
| (2)† | 745 | 3.1 | 785 | 3.1 | 740 | 3.9 | 860 | 4.4 | 945 | 4.0 | 950 | 3.8 | 850 | 4.4 | |
| JW (1) | 565 | 1.5 | 635 | 2.7 | 740 | 3.0 | 645 | 2.5 | 880 | 1.8 | 700 | 2.4 | 495 | 1.4 | Normal subject |
| (2)† | 485 | 0.6 | 510 | 1.2 | 500 | 0.7 | 545 | 0.7 | 590 | 0.7 | 680 | 1.4 | 655 | 0.3 | |
| RGH (1) | 665 | 5.3 | 800 | 5.1 | 825 | 6.2 | 1,055 | 5.6 | 1,220 | 6.8 | 1,255 | 2.8 | 970 | 5.3 | Normal subject |
| (2)† | 1,000 | 5.6 | 990 | 5.4 | 955 | 5.7 | 1,035 | 5.8 | 1,545 | 6.2 | 1,563 | 5.3 | 1,590 | 5.6 | |
| (3)‡ | 735 | 3.7 | 810 | 4.1 | 915 | 4.1 | 945 | 4.1 | 1,150 | | 1,005 | 7.3 | 725 | 4.6 | |
| (4)§ | 785 | 4.6 | 735 | 4.6 | 720 | | 815 | 1.4 | 865 | 2.7 | 885 | 3.2 | 815 | | |
| LT (1) | 830 | | 880 | | | | 1,030 | | 1,145 | | 1,135 | | 835 | | Normal subject |
| (2) | 837 | | 1,029 | | 959 | | 1,144 | | 1,227 | | 1,332 | | 1,046 | | |
| FM (1) | 720 | 5.9 | 770 | 6.6 | 735 | 6.8 | 920 | 5.9 | 790 | 5.9 | 970 | 6.3 | 680 | 5.4 | Normal subject |
| (2) | 680 | 6.6 | 870 | | | | 855 | 8.0 | 950 | 6.3 | | | 705 | 5.3 | |
| RF (1) | 635 | 2.1 | 910 | 2.0 | 965 | 2.2 | 1,035 | 2.1 | 1,145 | 4.6 | 1,200 | 1.5 | 740 | 2.2 | Normal subject |
| (2) | 740 | 2.1 | 965 | 1.8 | 975 | 2.2 | 1,170 | 2.2 | 1,275 | 1.9 | 1,390 | 5.2 | 730 | | |
| PT | 530 | 0.5 | 560 | 0.8 | 795 | 0.8 | 845 | 0.7 | 920 | 2.1 | 1,050 | 0.5 | 545 | 0.5 | Bilateral adrenalectomy |
| WB | 970 | 4.6 | 1,025 | 6.5 | 1,040 | 5.6 | 1,055 | | 955 | 5.1 | 985 | 3.7 | | | Primary autonomic insufficiency—subject fainted and tilt terminated at 18 min |

* FFA = free fatty acids; NE = norepinephrine.
† Five mg phentolamine administered i.v. prior to tilting.
‡ Repeat tilt procedure one month after first procedure.
§ Ten mg phentolamine administered i.v. prior to tilting.

control concentrations within 10 minutes. Plasma NE concentrations rose at least 1 μg per L plasma in each case after tilting (mean increment above control, 1.8 μg per L plasma), but showed no consistent temporal relationship between peak FFA response and peak circulating NE concentration.

The effect of phentolamine methanesulfonate on the FFA response in three normal individuals is shown in Figure 1B. In each case the FFA rise after phentolamine was depressed as compared with that of the first tilt. A rise in plasma NE concentration occurred in response to tilting both before (mean increase 2.0 μg per L) and after (mean increase 0.7 μg per L) phentolamine. The subject studied a second time (one month later) with 10 mg of phentolamine showed a greater depression of the FFA response than that which occurred with 5 mg of phentolamine.

The results of two successive tilts without receiving phentolamine prior to the second tilt are shown in Figure 1A. In each case the plasma FFA response during the second tilt exceeded that of the first.

The plasma FFA response to tilting in a patient with bilateral adrenalectomy for Cushing's disease two years previously, and now receiving 50 mg of cortisone daily, is shown in Figure 1C. The FFA response was found to be close to the mean response of the normal individuals. The postadrenalectomy patient showed a normal rise in plasma NE (1.6 μg per L) after tilting.

The patient with primary autonomic insufficiency showed essentially no increase in plasma FFA concentration in response to tilting (Figure 1C). His blood pressure gradually fell during the upright tilt and at 18 minutes he fainted. On two previous tilt studies his plasma NE increment was found to be less than 1 μg per L (9).

## DISCUSSION

It is well established that in the fasting state plasma FFA serves as a transport form of fat available for oxidative metabolism (10). In the fasting individual the adipose tissue is the major source of plasma FFA and it represents a potential pool of readily mobilizable substrate for energy requirements. The results reported here indicate that FFA can be mobilized in response to orthostatic stress. The FFA rise accompanies the hemodynamic responses and the increase in circulating NE concentrations and would seem to result from increased neuroadrenergic activity. An obligatory role of the adrenal medulla in mediating the FFA response to tilting is ruled out by the normal response to tilting in the postadrenalectomy patient. This indicates that the FFA mobilization secondary to tilting is not dependent upon the release of adrenal medullary hormones. The normal NE response to tilting in the patient after bilateral adrenalectomy indicates that the increase in circulating NE concentration after tilting is, for the most part, a "spill-over" of NE released at adrenergic nerve endings. The rise in circulating NE concentration is probably a reflection of neuroadrenergic activity. The possibility that the elevation in plasma NE concentration after tilting may represent decreased destruction rather than increased production of NE is not ruled out by these experiments, but this seems unlikely in view of the reported release of NE by the adrenergic nerves after various forms of stimulation (11).

The fact that the plasma FFA response during the second of the two successive tilts was greater than the response in the first tilt indicates that the decrease in the FFA response after phentolamine was not due to fatigue of the mobilization mechanism by two successive tilts.

The blocking action of phentolamine and the markedly diminished response in a patient with autonomic insufficiency suggest that mobilization of FFA from adipose tissue under the stimulus of orthostatic stress requires an intact adrenergic nerve supply and an available receptor site in adipose tissue. These results are in agreement with findings of others that phentolamine *in vitro* will block the release of FFA from rat epididymal fat pads by epinephrine (3) and that the mobilization of FFA may be actuated by emotional arousal and be depressed by ganglionic blocking agents (12).

From the evidence presented it would appear that neuroadrenergic stimulation in man is associated with an increase in plasma free fatty acid concentration. This supports the conception that the autonomic nervous system plays a significant role in the mobilization of free fatty acid in man.

SUMMARY

1. Plasma free fatty acid concentrations increased in normal fasting individuals in response to neuroadrenergic activity stimulated by the orthostatic stress of a 60° upright tilt.

2. This response was present in a patient following bilateral adrenalectomy but was greatly diminished in a patient with primary autonomic insufficiency.

3. The response of plasma free fatty acids to tilting can be diminished by the administration of an adrenolytic agent, phentolamine.

4. These results support the concept that the autonomic nervous system plays a significant role in the mobilization of free fatty acid in man.

REFERENCES

1. Wertheimer, E., and Shapiro, B. The physiology of adipose tissue. Physiol. Rev. 1948, **28**, 451.
2. White, J. E., and Engel, F. L. A lipolytic action of epinephrine and norepinephrine on rat adipose tissue *in vitro*. Proc. Soc. Exp. Biol. (N. Y.) 1958, **99**, 375.
3. Schotz, M. C., and Page, I. H. Effect of norepinephrine on plasma nonesterified fatty acids (NEFA) and the release of NEFA from rat epididymal adipose tissue. Fed. Proc. 1959, **18**, 139.
4. Goldfien, A., and Havel, R. J. The effects of norepinephrine and epinephrine on unesterified fatty acid metabolism. J. clin. Invest. 1959, **38**, 1007.
5. Hickler, R. B., Wells, R. E., Jr., Tyler, H. R., and Hamlin, J. T., III. Plasma catechol amine and electroencephalographic responses to acute postural change. Amer. J. Med. 1959, **26**, 410.
6. Dole, V. P. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. J. clin. Invest. 1956, **35**, 150.
7. Weil-Malherbe, H., and Bone, A. D. Chemical estimation of adrenaline-like substances in the blood. Biochem. J. 1952, **51**, 311.
8. Aronow, L., and Howard, F. A. Improved fluorometric technique to measure changes in adrenal epinephrine-norepinephrine output caused by veratrum alkaloids. Fed. Proc. 1955, **14**, 315.
9. Hickler, R. B., Thompson, G. R., Fox, L. M., and Hamlin, J. T., III. Successful treatment of orthostatic hypotension with 9-alphafluorohydrocortisone. New Engl. J. Med. 1959, **261**, 788.
10. Fredrickson, D. S., and Gordon, R. S., Jr. Transport of fatty acids. Physiol. Rev. 1958, **38**, 585.
11. von Euler, U. S. Noradrenaline. Springfield, Ill., Charles C Thomas, 1956.
12. Bogdonoff, M. D., Weissler, A. M., Merritt, F. L., Jr., Harlan, W. R., and Estes, E. H., Jr. The role of the autonomic nervous system in human lipid metabolism. J. clin. Invest. 1959, **38**, 989.

# HYPOCATALASEMIA: A NEW GENETIC CARRIER STATE *

By SHIGEO TAKAHARA,† HOWARD B. HAMILTON,‡ JAMES V. NEEL,‡§ THOMAS Y. KOBARA,‡ YOSHIO OGURA † AND EDWIN T. NISHIMURA ‡‖¶ WITH THE TECHNICAL ASSISTANCE OF KIYOKO OZAKI ‡ AND KYOKO ITO ‡

*(From Okayama University Medical School, Okayama, Japan, and the Atomic Bomb Casualty Commission, Hiroshima-Nagasaki, Japan)*

(Submitted for publication September 21, 1959; accepted December 4, 1959)

Acatalasemia, a rare congenital abnormality characterized by an apparent lack of the enzyme catalase, was first discovered by Takahara and Miyamoto in 1947 (1, 2). The lack of catalase activity in this disease was initially noted in whole blood, but subsequent studies of the tissues of other organs such as the nasal and oral cavities, the pharynx, bone marrow and liver have revealed a similar absence of activity for this enzyme (3–5). Since the initial description of this disease, 38 cases in 17 families have been found and reported in Japan up to April 1959 (6–17); at this writing no reports of acatalasemia have appeared from any other countries of the world.

Shortly after the discovery of acatalasemia it became apparent that the condition probably resulted from homozygosity for a recessive gene (18). Because of the widespread interest in the biochemical identification of genetic carrier states, we undertook a study of persons presumed to be heterozygous for this gene. A recent preliminary report from this laboratory has shown that while certain members of the families of acatalasemic subjects have normal catalase activity in the peripheral blood, others have low values (hypocatalasemia) (19). It was shown further that individuals who, from their position in the family, should be genetic carriers (heterozygotes) are hypocatalasemic. The purpose of this paper is to review the clinical aspects of this disease and its carrier state, to describe in some detail the biochemical procedures utilized in the estimation of catalase values in hypocatalasemics, and to present certain genetic considerations concerning hypo- and acatalasemia.

## METHODS OF STUDY

The study team was divided into *a*) a field investigation unit composed of two members of the Okayama University group and *b*) four members of the Atomic Bomb Casualty Commission. The field investigation group was responsible for the arrangement of all contacts with the families under study, for reviewing the genealogical relationship among their members, and for the physical examinations. This group collected all blood specimens and delivered them under refrigeration to the laboratory in Hiroshima within a few hours after obtaining the samples. The families investigated are those in which acatalasemia cases were previously detected, and they have been the subject of earlier reports (1, 6, 15, 16).

The standardization and the establishment of normal blood catalase values were performed on subjects at the medical clinic of the Atomic Bomb Casualty Commission in Hiroshima and Nagasaki.[1] The individuals tested were of both sexes and ranged in age from 11 years to over 70.

Blood, collected in a dry syringe, was immediately heparinized, and unless assay was performed shortly

* Presented in part at the Japanese Genetic Society Meeting, Nagoya, Japan, October 20, 1958. These data were collected under the auspices of the Department of Otorhinolaryngology of Okayama University Medical School, Okayama, Japan; and the Atomic Bomb Casualty Commission, a field research agency of the National Academy of Sciences, National Research Council of the United States, with financial support from the U. S. Atomic Energy Commission, administered in cooperation with the National Institutes of Health of the Japanese Ministry of Health and Welfare.

† Department of Otorhinolaryngology, Okayama University Medical School, Okayama, Japan.

‡ Atomic Bomb Casualty Commission, Hiroshima-Nagasaki, Japan.

§ Present address: Department of Human Genetics, University of Michigan Medical School, Ann Arbor, Mich.

‖ Present address: Department of Pathology, Northwestern University Medical School, Chicago, Ill.

¶ Senior Research Fellow, United States Public Health Service.

[1] Although no significant difference was noted between the mean catalase value among those exposed and those not exposed to significant radiation, the former have been excluded from the normal control group.

thereafter, was kept under refrigeration. Specimens collected from various areas in Japan were shipped to the laboratory in iced vacuum containers, since storage at 4° for 3 or 4 days was found to have no significant effect on catalase activity.

Hemoglobin was determined by the cyanomethemoglobin method of Drabkin (20) as modified by Crosby, Munn and Furth (21), using a Coleman Junior spectrophotometer.

The assay of catalase activity, based on methods developed by von Euler and Josephson (22), Bonnichsen, Chance and Theorell (23), and Herbert (24), was performed as follows: an appropriate aliquot of whole blood was diluted to 500 ml with distilled water to make a final hemoglobin concentration of 0.14 g per L and mixed gently. Using a blow-out pipet, 1 ml of the diluted hemoglobin solution was placed *immediately* in each of 5 test tubes containing a 5 ml solution of 0.01 M hydrogen peroxide and 0.01 M phosphate buffer, pH 6.8, prewarmed to 37° C in a constant temperature water bath. After exactly 15 seconds' incubation, timed by a stopwatch, the reaction in one tube was stopped by the immediate addition of approximately 2 ml of 2 N sulfuric acid from a beaker; similarly, acid was added successively to three of the remaining tubes at intervals of 30, 45, and 60 seconds. The fifth tube, to which acid was added *before* the diluted blood sample, was used to determine the initial hydrogen peroxide concentration; the peroxide concentration in each tube was determined by titration with 0.005 N potassium permanganate. Under these conditions the disappearance of peroxide follows first order kinetics, and the velocity constant $k_1$, which may be used as a measure of catalase activity, is calculated for each time interval from the equation

$$k_1 = \frac{1}{t} \log_{10} \frac{x_0}{x}$$

where $x_0$ is the initial peroxide concentration expressed as milliliters of permanganate and x is the concentration at time t. In this report, the mean of the four values times $10^5$, hereinafter referred to as $K_{cat}$, is used to express erythrocyte catalase activity. Table I shows the data from a typical determination.

The four $K_{cat}$ values for each determination agree closely and do not increase with increasing reaction times up to 1 minute. Analysis of 25 sets of four such readings (selected randomly) yielded a standard deviation of 0.11 $K_{cat}$ units for a single reading within any set of four, the standard error of the mean of the four readings which is taken as the $K_{cat}$ value for the individual thus being 0.055 $K_{cat}$ units. Other sources of laboratory error are minimized by careful control of the conditions under which the assay is performed. As noted by Maehly and Chance (25), a first order reaction is obtained, provided *a*) a short reaction time is employed, *b*) a low substrate concentration and a low ionic strength buffer are used, and *c*) the assay is carried out immediately after final dilution of the blood sample. Temperature in a range between 0° and 60° C has no significant effect on $K_{cat}$ values and 37° was found to be suitable and convenient for the present assay. Under these conditions, catalase activity was shown to be linearly related to enzyme concentration using purified human erythrocyte catalase prepared according to the method of Herbert and Pinsent (26). There is also a linear relationship between catalase activity and hemoglobin concentration in normal individuals, a finding previously reported by Funaki (27) and Miller (28), using different assay methods.

TABLE I

*Data from a typical catalase activity assay*

| Time | $H_2O_2$ remaining | $\log_{10} x_0/x$ | $K_{cat}$ |
|---|---|---|---|
| *sec* | *ml $KMnO_4$* | | |
| 0 | 9.87 | 0 | |
| 15 | 8.07 | 0.0874 | 5.83 |
| 30 | 6.55 | 0.1781 | 5.94 |
| 45 | 5.37 | 0.2643 | 5.88 |
| 60 | 4.36 | 0.3548 | 5.91 |
| | | | Mean 5.89 |

In addition to estimating the error of a single reading at 15, 30, 45, and 60 seconds, the variation between duplicate assays on the same sample was also studied. For 18 such duplicate determinations, the standard deviation of the difference between the means of the four readings for each pair was found to be 0.04. The two sources of laboratory variation for which explicit estimates are provided combine to produce a standard error of 0.07 for a $K_{cat}$ determination on an individual. This estimate of laboratory error may be compared with the value 0.73 calculated as the standard deviation of the distribution of individual $K_{cat}$ values, including *both* laboratory error and individual variation.

In establishing the range of normal values, it was found that neither age nor sex appeared to have any significant influence on the level of blood catalase activity. Daily and weekly fluctuations in normal individuals do not seem remarkable; serial determinations in both males and females showed variations not exceeding 5 per cent.

*Clinical considerations.* There is no gross difference in appearance between the blood of an acatalasemic and that of a normal individual. However, upon the addition of $H_2O_2$, in contrast to normal blood which remains bright red and foams exuberantly due to the action of erythrocyte catalase, acatalasemic blood immediately turns a black-brown color as methemoglobin is formed, and no foaming occurs. On standing, the black-brown color gradually fades and about 30 minutes later a flocculent yellow-white precipitate containing denatured protein settles out, leaving a clear colorless supernatant fluid containing propentdyopent, the final breakdown product of hemoglobin destruction by hydrogen peroxide (6).

Clinically, approximately 60 per cent of the people with acatalasemia thus far recognized have developed in childhood a peculiar progressive gangrenous disease within

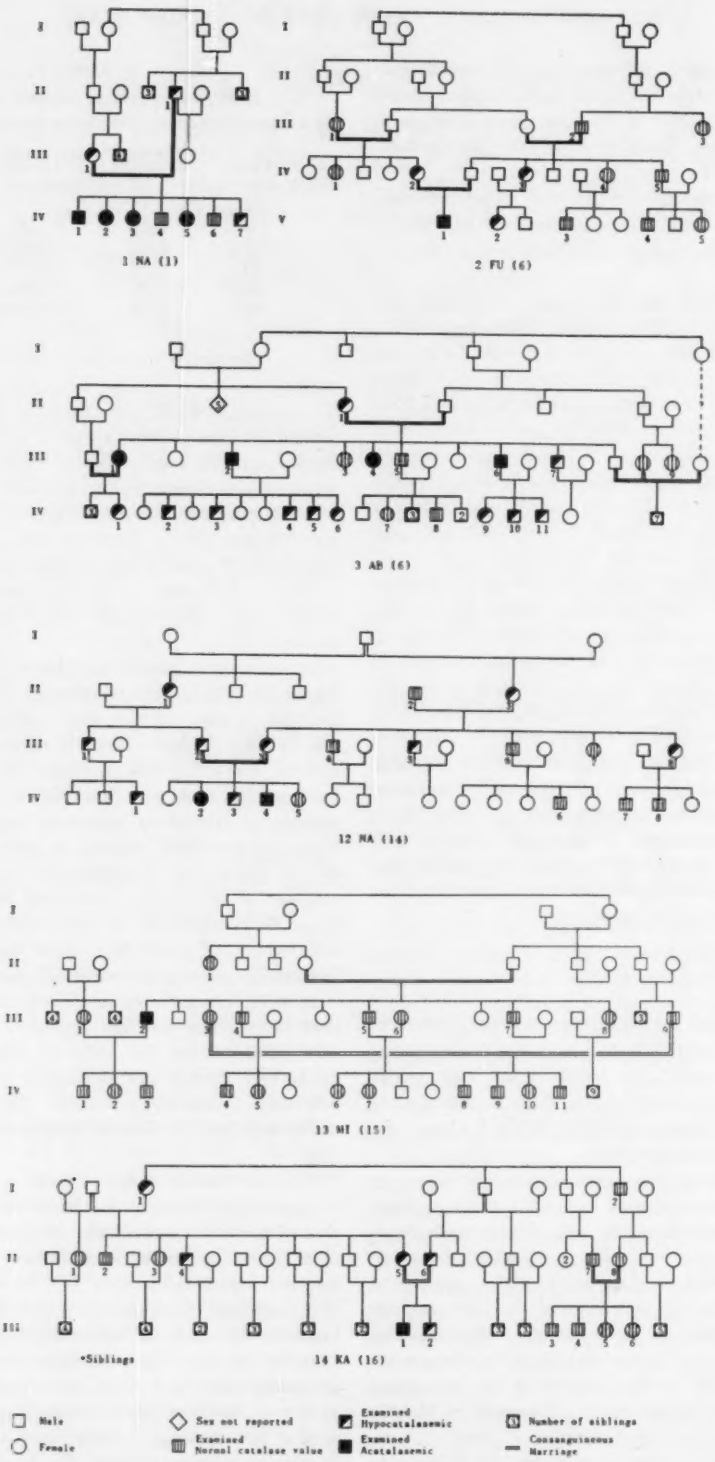



FIG. 1. PEDIGREES OF FAMILIES WITH ACATALASEMIA IN WHICH HYPOCATALASEMIA HAS BEEN STUDIED. The families are numbered according to Takahara and associates (*29*, 30). Each person studied may be located in the pedigree by a Roman numeral indicating generation and an Arabic number indicating position within the generation. Thus, 1 NA IV-2 is the second individual in the fourth generation of family 1 NA.

the oral cavity (Takahara's disease). In many cases this disease develops at the roots of the teeth but, in some rare instances, the original site may be the crypt of the tonsil. The disease that develops from the roots of the teeth may be classified into three types according to the symptoms: mild, moderate or severe.

In the mild type, repeatedly recurring ulcers with pain are noted in the dental alveoli. In the moderate type, alveolar gangrene develops, and atrophy and recession of the alveolar bone expose much of the neck of the teeth, which loosen and fall out spontaneously. In the severe type, the gangrene of bone or of the soft tissues around the maxilla, mandible or teeth is progressive and presents a picture of widespread destruction. Of the three types, the moderate is the most common (about 50 per cent) while the mild and severe types are seen with about equal frequency.

Takahara (6) believes that the pathogenesis of the oral ulceration may be related to the fact that various bacteria (hemolytic streptococci among them) normally present in the gingival mucosa, may under favorable circumstances rapidly proliferate so that excessive amounts of $H_2O_2$ are produced. Normal individuals have the capacity to destroy this product, but persons with acatalasemia cannot, and a vicious circle is established. $H_2O_2$ oxidizes the hemoglobin of the red blood cells, thereby depriving the tissues of the oxygen normally carried by the erythrocytes; the hydrogen peroxide and bacteria invade the surrounding tissues, general tissue breakdown occurs, and gangrene supervenes. Treatment consists of extraction of the diseased teeth or tonsil and the use of antibiotics to control the harmful effects of the offending bacteria. There appears to be no difference in the speed of wound healing in comparison with normal individuals, once treatment has been instituted.

As noted above, about 25 per cent of the acatalasemic patients are asymptomatic, and never develop any form of oral disease. Further, even those who have had some form of disease of the oral cavity in their childhood are in other respects no different from normal people, and apparently after puberty even those individuals who had the severe type of oral disease become symptom-free. Among the 12 cases under observation by Takahara for 10 years or more, aside from showing various manifestations of oral disease in childhood as described above, none has developed any particularly serious disease and all are alive and well at this time.

*Description of families studied.* Thus far, six-kindreds have been investigated in detail. These are depicted in Figure 1. The numbering of the families corresponds to that employed by Takahara, Doi and Ogura in other reports devoted to a tabulation of all acatalasemics known to date (29, 30). In four kindred (1 NA, 2 FU, 3 AB, and 13 MI), the propositus (IV-2, V-1, III-2, and III-2, respectively) was an individual with the typical signs and symptoms of acatalasemia as described in the preceding section. In the remaining two kindreds, acatalasemia was asymptomatic. In kindred 12 NA, acatalasemia was discovered in the propositus, IV-2, when $H_2O_2$ was used in a surgical procedure not involving the oral cavity. In kindred 14 KA, the propositus, III-1, was suspected of having the condition when $H_2O_2$ was employed in the treatment of pustular eruptions, a suspicion later confirmed by appropriate studies.

## RESULTS OF LABORATORY STUDIES

As noted under "Methods," normal standards were established from studies on 259 individuals undergoing evaluation in the Medical Clinic of the Atomic Bomb Casualty Commission (ABCC). The distribution of $K_{cat}$ values among these controls is well approximated by a normal probability curve of mean 5.38 and standard deviation 0.73. The 95 per cent confidence interval of the means is 5.29 to 5.47. The range of control values is from 3.90 to 7.47. One would expect only 2.5 per cent of normal values to fall below the mean minus 2 SD (3.92 $K_{cat}$ units) and only 0.15 per cent below the mean minus 3 SD (3.19 units).

The actual $K_{cat}$ values for the 99 members of the six acatalasemic families studied in detail are shown in Table II; the column "pedigree position" refers to the detailed pedigrees in Figure 1. When the 99 values are arrayed in order of magnitude, 55 are found to lie well within the range (3.90 to 7.47) of ABCC clinic controls and within the range of the observed control mean $\pm$ 2 SD (3.92 to 6.84); the lowest of the 55 values is 3.96. The next value in the array is 2.87, which lies more than 3 SD below the observed control mean. There are 31 values in the range 2.87 to 1.48. All other individuals are acatalasemic, with zero $K_{cat}$ values. Thus it appears that there are at least three different groups of individuals within the acatalasemic families: the essentially normal, the hypocatalasemic, and the acatalasemic. The means, ranges, and standard deviations for the $K_{cat}$ values of these three groups, as well as for the controls, are shown in Table III. The hypocatalasemic values are definitely outside the normal range. However, when the 55 values *within* the normal range are compared with the 259 ABCC clinic controls, it is found that the means of 4.97 and 5.38 do differ significantly ($p < 0.01$). The discrepancy is thus not readily attributable to chance or to sampling variation. On the average the analysis of the blood specimens from the families with acatalasemia was delayed some 24 hours longer than the ABCC control specimens;

TABLE II

*Data for families studied in detail*

| Family | Pedigree position | $K_{cat}$ | Family | Pedigree position | $K_{cat}$ | Family | Pedigree position | $K_{cat}$ | Family | Pedigree position | $K_{cat}$ | Family | Pedigree position | $K_{cat}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | II-1 | 2.62 | | II-1 | 2.87 | | II-1 | 2.08 | | II-1 | 5.05 | | I-1 | 1.49 |
| | III-1 | 1.89 | | III-1 | 0.0 | | II-2 | 4.05 | | III-1 | 6.47 | | I-2 | 4.11 |
| | IV-1 | 0.0 | | III-2 | 0.0 | | II-3 | 2.16 | | III-2 | 0.0 | | II-1 | 5.41 |
| | IV-2 | 0.0 | | III-3 | 5.40 | | III-1 | 2.25 | | III-3 | 5.77 | | II-2 | 4.99 |
| 1 NA | IV-3 | 0.0 | | III-4 | 0.0 | | III-2 | 2.55 | | III-4 | 4.03 | | II-3 | 5.39 |
| | IV-4 | 4.41 | | III-5 | 6.20 | | III-3 | 2.19 | | III-5 | 4.87 | | II-4 | 2.55 |
| | IV-5 | 0.0 | | III-6 | 0.0 | | III-4 | 5.10 | | III-6 | 5.64 | | II-5 | 1.50 |
| | IV-6 | 4.77 | | III-7 | 2.25 | | III-5 | 2.31 | | III-7 | 4.86 | | II-6 | 2.00 |
| | IV-7 | 2.21 | | III-8 | 5.54 | | III-6 | 4.13 | | III-8 | 4.04 | 14 KA | II-7 | 4.72 |
| | | | | III-9 | 5.02 | 12 NA | III-7 | 5.83 | | III-9 | 5.04 | | II-8 | 4.89 |
| | III-1 | 4.74 | 3 AB | IV-1 | 2.31 | | III-8 | 2.33 | 13 MI | IV-1 | 5.03 | | III-1 | 0.0 |
| | III-2 | 4.44 | | IV-2 | 2.10 | | IV-1 | 1.55 | | IV-2 | 4.20 | | III-2 | 1.48 |
| | III-3 | 4.78 | | IV-3 | 2.13 | | IV-2 | 0.0 | | IV-3 | 5.63 | | III-3 | 5.95 |
| | IV-1 | 4.69 | | IV-4 | 2.30 | | IV-3 | 1.57 | | IV-4 | 4.82 | | III-4 | 4.88 |
| | IV-2 | 1.98 | | IV-5 | 2.23 | | IV-4 | 0.0 | | IV-5 | 3.96 | | III-5 | 4.89 |
| | IV-3 | 2.12 | | IV-6 | 2.41 | | IV-5 | 4.83 | | IV-6 | 4.49 | | III-6 | 4.52 |
| 2 FU | IV-4 | 4.74 | | IV-7 | 5.40 | | IV-6 | 4.21 | | IV-7 | 4.41 | | | |
| | IV-5 | 4.73 | | IV-8 | 5.31 | | IV-7 | 5.56 | | IV-8 | 4.05 | | | |
| | V-1 | 0.0 | | IV-9 | 2.32 | | IV-8 | 6.07 | | IV-9 | 6.19 | | | |
| | V-2 | 2.45 | | IV-10 | 2.28 | | | | | IV-10 | 5.49 | | | |
| | V-3 | 4.71 | | IV-11 | 2.40 | | | | | IV-11 | 4.79 | | | |
| | V-4 | 5.42 | | | | | | | | | | | | |
| | V-5 | 5.07 | | | | | | | | | | | | |

the possibility that a slight loss of enzyme activity with age, not detected on the standardization studies, contributes to the observed difference must be considered.

*Genetic considerations.* The "recessive" inheritance of acatalasemia, first postulated by Takahara, Sato, Doi and Mihara (18), has been amply confirmed by all subsequent family studies. In the 17 segregating sibships thus far reported, the ratio of affected to normal is 36 to 57, with males and females affected in equal numbers. This corresponds perfectly to expectation for a recessive trait, on the assumption of ascertainment through a single affected individual for each segregating sibship—an assumption which, while not strictly justified, provides a reasonable working approach to the data.

TABLE III

*$K_{cat}$ values in controls and in acatalasemic families*

| Source and type of patients | Number of individuals | $K_{cat}$ Values | | |
|---|---|---|---|---|
| | | Mean | SD | Range |
| ABCC* clinic controls | 259 | 5.38 | 0.73 | 3.90–7.47 |
| Acatalasemic families: | | | | |
| "Normals" | 55 | 4.97 | 0.61 | 3.96–6.47 |
| Hypocatalasemics | 31 | 2.17 | 0.35 | 1.48–2.87 |
| Acatalasemics | 13 | 0 | | |

* Atomic Bomb Casualty Commission.

With respect to hypocatalasemia, a detailed consideration of the pedigrees shown in Figure 1 (ignoring for the moment family 13 MI) brings out a number of genetically significant facts: *a*) hypocatalasemic values have been found in every instance in which it has been possible to study one or both parents of an acatalasemic individual; *b*) the children of unrelated parents, one an acatalasemic and the other presumably normal, are hypocatalasemic; *c*) the siblings of parents of affected individuals are found to be either normal or hypocatalasemic ; *d*) the sex distribution among the hypocatalasemics is 17 males and 13 females. In three families (2, 12, 14) it has been possible to trace the defect for three generations. All these findings indicate that hypocatalasemia is the heterozygous carrier state for the gene which, when homozygous, is responsible for acatalasemia.

Family 13 MI is anomalous with respect to the observations noted above. In this family there is one acatalasemic; all other members in the kindred who were tested (among whom are the affected individual's children, several of his siblings and their children, including the offspring of a consanguineous marriage between a sister and cousin) were found to have normal blood catalase activity.

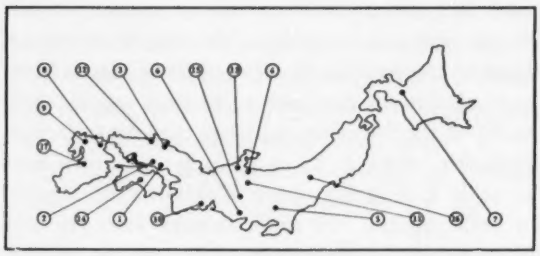


FIG. 2. DISTRIBUTION OF 17 ACATALASEMIC FAMILIES IN JAPAN.

In this group, the acatalasemic individual and several others were retested six months later with similar results. Under carefully controlled conditions, such an observation would seem best interpreted as indicating a genetic heterogeneity of the acatalasemic phenotype, with the resultant conclusion that there are two (or more) recessively inherited types of acatalasemia, one associated with a definite decrease in catalase activity in heterozygotes, the other not. However, the uncertainties of research in human genetics are such that until satisfactory corroboration of this finding, complete with blood group studies, is available, we shall consider this disease as a single entity.

The clinical significance, if any, of hypocatalasemia remains to be elucidated. There are no obvious ill effects. More specificially and carefully directed questioning has brought out no unusual susceptibility to disease involving the oral cavity. Despite the rarity of (homozygous) acatalasemics, the frequency of (heterozygous) hypocatalasemics is sufficient to make studies on the clinical and subclinical manifestations of this entity both feasible and, in view of the poorly understood functions of this enzyme, desirable. Thus, Figure 2 illustrates the location of the 17 Japanese families in whom acatalasemia has been reported. Neglecting for the moment the earlier discussed possibility that acatalasemia is genetically heterogeneous, a possibility to which we shall return later, then Figure 2 reveals that acatalasemia is not restricted to any one portion of Japan, with the corollary that the responsible gene is one of considerable antiquity which has become well dispersed throughout the country. Under these circumstances, the frequency of the gene can be *approximated* from the well known relationship

$$q = \frac{c(1 - k)}{16\,k - 15\,c - ck},$$

where q = the frequency of the recessive gene responsible for acatalasemia, k = the proportion of first cousin marriages among the parents of affected persons, and c = the proportion of first cousin marriages among the population as a whole.

In the determination of k, the authors of case reports on material not available to us have been requested to check their earlier observations concerning consanguinity; insofar as rechecks have been possible, the initial reports have been confirmed. Figure 3 summarizes the facts concerning consanguineous parentage in the material of other investigators of this disease. Our own ma-

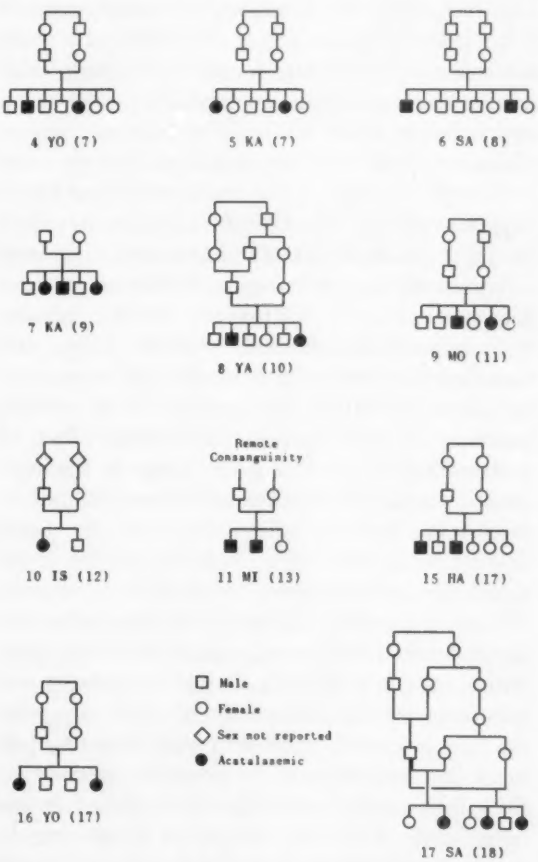



FIG. 3. ABBREVIATED PEDIGREES OF FAMILIES WITH ACATALASEMIA NOT STUDIED FOR HYPOCATALASEMIA.

terial has been carefully investigated on this point. From Figures 2 and 3 it can be seen that, for the 17 sibships so far recorded as segregating for acatalasemia, consanguinity of some degree has been recorded in every instance but one. Ten of the 17 marriages involve first cousins, while two involve not only first cousins but additional degrees of relationship. The treatment of these latter in the present context is uncertain, simply because most studies on the frequency of consanguineous marriage fail to record the details in such instances, and control population figures are not available. Disregarding these two marriages, the value of k becomes $10/17 = 0.59$. The value of c is much higher for Japanese populations than for European or American, approximating 0.06 (31). The value of $q$ is thus approximately 0.003. The frequency of heterozygotes is given by the expression $2q\ (1-q)\ (1-\alpha)$, while the frequency of homozygotes is $\alpha q + (1-\alpha)q^2$, where $\alpha$ represents the mean coefficient of inbreeding for the individuals comprising the population. In Japan, the value of $\alpha$ approximates 0.004 (31). The frequency of homozygotes with manifest disease should thus approximate 0.000021, while the frequency of heterozygous carriers is 0.005958. In a country such as Japan, with 88,000,000 inhabitants, it would follow from the foregoing calculations that there are approximately 500,000 to 550,000 persons with hypocatalasemia and 1,800 to 1,900 with acatalasemia (assuming a normal life expectancy for both groups). The use of these various formulae is based on the assumption that all acatalasemia is due to a gene change at the same genetic locus, and the further assumption of an essentially uniform distribution of the gene throughout Japan. Since it is the history of genetics that what appeared at first to be discrete clinical and genetic entities have repeatedly been shown to be heterogeneous, and since the distribution of a rare gene in a stable population can only approximate uniformity, these assumptions can only be justified on the grounds that they permit a first estimation of the frequency of these entities that cannot readily be accomplished in any other way. A further assumption underlying the use of these formulae is that of genetic equilibrium in the population. In view of the likelihood that inbreeding levels in Japan have been decreasing over the past century, here too is a cause to view the foregoing calculations with some reservations. Most of the sources of error in these assumptions and calculations are such as to give inflated estimates of the frequency of hypo- and acatalasemia. However, even if, as is quite possible, the true value of q is of the order of 0.0015, the number of homozygotes still approximates 700, or, with half normal life expectancy, 350, and the number of heterozygotes, 260,000. If, as discussed earlier, there are actually two types of acatalasemia, only one of which is associated with an identifiable carrier state, then the frequency of hypocatalasemia is of course lower. The development of a rapid screening method for the detection of hypocatalasemia would make it possible to check directly on the correctness of the assumptions which have entered into this calculation of carrier frequencies.

Since the discovery of acatalasemia in Japan, there has been a vigorous, continuing search for new cases of the disease. On this basis, there would seem to be a real discrepancy between the number of cases estimated by gene frequency techniques to occur in Japan, and the number actually observed. A clue to this apparent discrepancy may be found in Takahara's clinical observation that some individuals with acatalasemia exhibit little in the way of ill effects. For instance, the discovery of acatalasemia in family 12 NA was quite fortuitous. Thus it is conceivable that in the majority of sibships in which one or more affected individuals occur, the condition is so mild in its manifestations as to be of no clinical significance, and hence it escapes detection.

There remains to be considered the apparently restricted distribution of acatalasemia. Although the discovery of this disease in Japan has sparked an enthusiastic quest for additional cases in that country which far exceeds the effort being expended in other countries, it seems unlikely that this is the entire answer to the lack of case reports from Europe or America. The possibility exists that the gene responsible for acatalasemia will be found to have just as restricted a distribution as genes responsible for hemoglobins C and N in the African Negro (32, 33). One inference from these somewhat analogous situations is that although mutation generally tends to be repetitive in nature, these particular traits owe their occurrence to mutations of great rarity, which by

chance, in some instances aided by selection, have became established in some populations but not in others. Only further research will tell whether acatalasemia is strictly a Japanese gene or whether, like the genetically controlled Diego factor, it will be found in all representative Mongolian populations.

## DISCUSSION

In recent years there has been increasing interest in the development of methods for the successful detection of the "carriers" of inherited diseases. By the application of appropriate laboratory tests to relatives of individuals who exhibit some biochemical abnormality as the expression of a hereditary disease, it is possible to detect clinically unapparent variations from normal that are clearly part of an altered genetic pattern. Thus far, laboratory tests, capable of contributing *with regularity* to the detection of the heterozygous carriers of nominally recessive genes, exist for approximately a dozen entities (34–37). However, for only two other diseases, the hereditary hemoglobinopathies and cholinesterase deficiency (38), does there appear to be the precision and ease of diagnosis of the carrier state that exists for acatalasemia. In most other instances, there appears to be substantial overlapping of results between carriers and normals.

The fact that the catalase values of heterozygotes are very nearly intermediate between those of acatalasemics and normal individuals has important implications from the standpoint of biochemical genetics. Although the molecular structure of catalase has not yet been elucidated, it seems reasonable to postulate that acatalasemia involves either an alteration in the amount of enzyme protein produced or an alteration in specificity. From the linearity of the relationship between the amount of active catalase present, and that, therefore, in individuals heterozygous for the acatalasemic gene, the amount of active enzyme protein seems to be very close to half, or slightly less than half, of normal.

The most nearly comparable situation in human genetics thus far explored in detail pertains to inherited variations in the hemoglobin molecule. Hemoglobins A, S, and C, whose production is under control of a series of three allelic genes, differ from one another with respect to only a single amino acid out of the 300 amino acids present in each of the two symmetrical half molecules which comprise human hemoglobin (39, 40). However, in individuals heterozygous for the gene responsible for hemoglobins S or C, only 30 to 40 per cent of the hemoglobin is of the abnormal type (41, 42). Individuals homozygous for either of these genes, with all the hemoglobin abnormal, have a significantly decreased hematopoietic reserve (43, 44). This leads to the conclusion that a probably quite *minor* change in a gene, so small as to result in a single amino acid substitution in a protein, may at the same time very significantly reduce what may roughly be termed "gene efficiency," i.e., as judged by the quantity of end product (45). Otherwise stated, if we envision hemoglobin as a fairly direct reflection of the catalytic activity of a gene or gene-product, then in the case of the hemoglobinopathies it would appear that a change so slight as to involve only a single amino acid in hemoglobin has very significantly impaired the catalytic effectiveness of that gene or gene-product. The same gene change seems to have both qualitative and quantitative results.

The situation in acatalasemia appears to be potentially different. The enzymatic activity of the heterozygote is no more than half of normal—taken at face value, actually 44 per cent of normal, although a large error attaches to this value of 44 per cent. From the results to date it is not clear whether in acatalasemia no protein corresponding to the catalase of the normal individual is being formed, or whether a closely related protein which, however, lacks enzymatic activity is elaborated. It may be possible by techniques currently available to distinguish between these possibilities. If it is the former, then the possibility that the remaining normal allele may be associated, under these circumstances, with less than half the normal quantity of gene product is a puzzling observation, since one must assume that the mutant gene not only results in the absence of the end product normally associated with this locus but also seemingly depresses the activity of its normal allele. If, on the other hand, the alternative of the production of an altered protein which was suggested above is correct, then acatalasemia may be a case in which a mutation is responsible

for a change in the structure of a protein, but it does not alter the "efficiency" of the gene involved, and indeed, if the data are taken at face value, may actually increase the efficiency.


## SUMMARY

A clinical, biochemical and genetic study has been made of a rare hereditary disease, acatalasemia, characterized by the absence of activity of the enzyme catalase from the blood and other tissues. Clinically, about half of the acatalasemics suffer from a peculiar type of oral gangrene (Takahara's disease) in childhood. This disease, sporadically distributed throughout Japan, is as yet unreported elsewhere in the world. To date 36 acatalasemics in 17 sibships are known. The high incidence of consanguineous marriage between the parents of the affected persons, the normality of the parents, and the ratio of affected to normal in segregating sibships indicate that the acatalasemic phenotype is recessively inherited.

Heterozygotes, described earlier for this disease, have now been verified, using a biochemical assay for red blood cell catalase activity; the mean activity ($K_{cat}$ in this report) of a normal control group is 5.38 units while that of the heterozygotes is 2.17, and no overlap in values between the two groups has been found. The identification of this carrier state would thus place the gene responsible for acatalasemia in the category of being incompletely recessive (or incompletely dominant). In one of the six families studied, no carrier state could be demonstrated. There are apparently no clinical manifestations in the heterozygotes.



## ACKNOWLEDGMENT

We gratefully acknowledge the assistance of Mr. Shoichi Ueda, Department of Statistics, ABCC, in the analysis of laboratory error and in establishing the presumptive normal range for $K_{cat}$ values.


## REFERENCES


1. Takahara, S., and Miyamoto, H. Three cases of progressive oral gangrene due to lack of catalase in the blood. (In Japanese.) Nihon Jibi Inkôka Gakkai Kaiho (J. Otorhinolaryngological Soc. Japan) 1948, **51**, 163.
2. Takahara, S., and Miyamoto, H. Clinical and experimental studies on progressive oral gangrene probably due to lack of catalase in the blood. (In Japanese.) Jibi Inkôka (Otolaryngology) 1949, **21**, 53.
3. Takahara, S., Mihara, S., Tsugawa, K., and Doi, M. Acatalasemia II. Contents of catalase in blood and tissues of men and animals. Proc. Japanese Academy 1952, **28**, 383.
4. Takahara, S. Progressive oral gangrene probably due to lack of catalase in the blood (acatalasaemia), report of nine cases. Lancet 1952, **2**, 1101.
5. Nakamura, H., Yoshiya, M., Kajiro, K., and Kikuchi, G. On "Anenzymia Catalasea" a new type of constitutional abnormality. Proc. Japanese Academy 1952, **28**, 59.
6. Takahara, S. Acatalasemia (lack of catalase in blood) and oral progressive gangrene. Proc. Japanese Academy 1951, **27**, 295.
7. Yoshiya, M. "Anenzymia Catalasea," a new type of constitutional abnormality. (In Japanese, with English summary.) Kôkûbyo Gakkai Zasshi (J. Stomatological Soc.) 1952, **19**, 18.
8. Yoshiya, M., and Mukai, T. A slight case of "Anenzymia Catalasea." Kôkûbyo Gakkai Zasshi (J. Stomatological Soc.) 1953, **20**, 277.
9. Hayashi, H., Ozaki, S., Ishizuka, T., and Ueda, H. Case report of catalase deficiency. (In Japanese.) Kôkûbyo Gakkai Zasshi (J. Stomatological Soc.) 1954, **3**, 254.
10. Kuhara, K., and Yamada, C. Two sibling cases of acatalasemia. (In Japanese.) Rinshô Shi-ka (Clinical Dentistry) 1954, **205**, 12.
11. Shirabe, K. Two cases of symptomless acatalasemia. (In Japanese, with English summary.) Jibi Inkôka, Otorhinolaryngology) 1956, **28**, 57.
12. Kitajiri, K., and Kuwahara, M. A case of acatalasemia. (In Japanese.) Nihon Jibi Inkôka Gakkai Kaiho (J. Otorhinolaryngological Soc. Japan) 1954, **57**, 97.
13. Takeuchi, K. Two symptomless cases of acatalasemia. (In Japanese, with English summary.) Jibi Inkôka (Otolaryngology) 1957, **29**, 121.
14. Kikuchi, H., Ogura, Y., and Doi, K. Two cases of acatalasemia complicated with nephritis. (In Japanese, with English summary.) Jibi Inkôka (Otolaryngology) 1958, **30**, 20.
15. Yonemaru, T., and Sanno, I. Three cases of acatalasemia. (In Japanese.) Jûzen Igakkai Zasshi (J. Jûzen Medical Soc.) 1958, **60**, 572.
16. Onishi, C., and Hisamochi, H. One case report of symptomless acatalasemia. (In Japanese.) Chiryô (Therapeutics) 1959, **41**, 771.
17. Tochihara, Y. Three cases of acatalasemia. (In Japanese.) Saikai Iho (Saikai Medical News) 1958, **119**, 3.
18. Takahara, S., Sato, H., Doi, M., and Mihara, S. Acatalasemia III. On the heredity of acatalasemia. Proc. Japanese Academy 1952, **28**, 585.
19. Nishimura, E. T., Hamilton, H. B., Kobara, T. Y., Takahara, S., Ogura, Y., and Doi, K. Carrier

state in human acatalasemia. Science 1959, **130**, 333.

20. Drabkin, D. L. Crystallographic and optical properties of human hemoglobin. A proposal for the standardization of hemoglobin. Amer. J. med. Sci. 1945, **209**, 268.
21. Crosby, W. H., Munn, J. I., and Furth, F. W. Standardizing a method for clinical hemoglobinometry. U.S. armed Forces med. J. 1954, **5**, 693.
22. von Euler, H., and Josephson, K. Catalase I. Justus Liebigs Ann. Chem. 1927, **452**, 158.
23. Bonnichsen, R. K., Chance, B., and Theorell, H. Catalase activity. Acta chem. scand. 1947, **1**, 685.
24. Herbert, D. Catalase from Bacteria (*Micrococcus lysodeikticus*) *in* Methods of Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York, Academic Press, 1955, vol. 2, p. 784.
25. Maehly, A. C., and Chance, B. The Assay of Catalases and Peroxidases *in* Methods of Biochemical Analysis, D. Glick, Ed. New York, Interscience Publishers, 1954, vol. 1, p. 357.
26. Herbert, D., and Pinsent, J. Crystalline human erythrocyte catalase. Biochem. J. 1948, **43**, 203.
27. Funaki, H. Thermal analysis of blood catalase reaction. Jap. J. Physiol. 1955, **5**, 183.
28. Miller, H. The relationship between catalase and haemoglobin in human blood. Biochem. J. 1958, **68**, 275.
29. Takahara, S., and Doi, K. Statistical observation on 35 cases of acatalasemia, which appeared in the literature. (In Japanese with English summary.) Nihon Jibi Inkôka Gakkai Kaiho (J. Otorhinological Soc. Japan) 1958, **61**, 1727.
30. Takahara, S., Ogura, Y., and Doi, K. Statistical study of acatalasemia: A review of thirty-eight cases appearing in the literature. Acta Med. Okayama. In press.
31. Neel, J. V., Kodani, M., Brewer, R., and Anderson, R. C. The incidence of consanguineous matings in Japan, with remarks on the estimation of comparative gene frequencies and the expected rate of appearance of induced recessive mutations. Amer. J. hum. Genet. 1949, **1**, 156.
32. Lehmann, H. Human haemoglobins. St Bart's Hosp. Rep. 1956, **60**, 237.
33. Neel, J. V. Human hemoglobin types: Their epidemiologic implications. New Engl. J. Med. 1957, **256**, 161.
34. Neel, J. V. The clinical detection of the genetic carriers of inherited disease. Medicine (Baltimore) 1947, **26**, 115.
35. Neel, J. V. The detection of the genetic carriers of inherited disease *in* Clinical Genetics, A. Sorsby, Ed. London, Butterworth, 1953, p. 27.
36. Hsia, D. Y-Y. The laboratory detection of heterozygotes. Amer. J. hum. Genet. 1957, **9**, 98.
37. Hsia, D. Y-Y. Inborn Errors of Metabolism. Chicago, Year Book Publishers, Inc., 1959.
38. Kalow, W., and Staron, N. On distribution and inheritance of atypical forms of human serum cholinesterase, as indicated by dibucaine numbers. Canad. J. Biochem. 1957, **35**, 1305.
39. Ingram, V. M. Gene mutations in human haemoglobin: The chemical difference between normal and sickle cell haemoglobin. Nature (Lond.) 1957, **180**, 326.
40. Hunt, J. A., and Ingram, V. M. Allelomorphism and the chemical differences of the human haemoglobins A, S and C. Nature (Lond.) 1958, **181**, 1062.
41. Wells, I. C., and Itano, H. A. Ratio of sickle-cell anemia hemoglobin to normal hemoglobin in sicklemics. J. biol. Chem. 1951, **188**, 65.
42. Neel, J. V., Wells, I. C., and Itano, H. A. Familial differences in the proportion of abnormal hemoglobin present in the sickle cell trait. J. clin. Invest. 1951, **30**, 1120.
43. Crosby, W. H., and Akeroyd, J. H. Limit of hemoglobin synthesis in hereditary hemolytic anemia. Amer. J. Med. 1952, **13**, 273.
44. Terry, D. W., Motulsky, A. G., and Rath, C. E. Homozygous hemoglobin C: A new hereditary hemolytic disease. New Engl. J. Med. 1954, **251**, 365.
45. Neel, J. V. Aspects of the genetic control of the structure of the hemoglobin molecule. Proc. Tenth Int. Congr. Genetics, 1959, **1**, 108.

# STUDY OF THE GENETIC AND EXTRA-GENETIC DETERMINANTS OF ALPHA-KETOSTEROID PRODUCTION IN MAN

By ATTALLAH KAPPAS* AND T. F. GALLAGHER

*(From the Division of Steroid Metabolism and Biochemistry, The Sloan-Kettering Institute for Cancer Research, New York, N. Y.; and the Department of Medicine and the Argonne Cancer Research Hospital,† of The University of Chicago, Chicago, Ill.)*

(Submitted for publication October 6, 1959; accepted December 4, 1959)

The present investigation was undertaken to evaluate the influence of heredity on steroid hormone production of man. The steroid patterns of adult male, dichorionic monozygotic triplets were examined in appropriate periods before, during and after adrenal stimulation with adrenocorticotropic hormone (ACTH). The results of this investigation provide evidence for the existence of a significant genetic determinant of the level of steroid hormone production and metabolism, as well as an important extra-genetic influence that can modify this factor. The nature of this extra-genetic influence is suggested from previous studies of physiological differences in twins.

## METHODS AND SUBJECTS OF STUDY

The subjects studied (F, H and J) were 25 year old, white, male, monozygotic triplets in excellent health. They were hospitalized during the course of the study, the essential details of which were as follows: 2 consecutive 3-day periods (Periods *1* and *2*) were followed by 3 1-day periods (Periods *3–5*) during which each subject received intravenously 20 U of ACTH dissolved in 500 ml of normal saline over 8 hours. Care was taken to assure that equal doses of ACTH from a common lot were given over the fixed periods of time. Injections were started after breakfast and the men were kept in bed during the infusions. Following the ACTH periods, 5 consecutive 1-day control studies were made (Periods *6–10*). Four years after the first studies, Subject H was re-examined during a control period, followed by 3 consecutive 1-day periods during which he received 20 U of ACTH intravenously each day, over an 8 hour interval.

Complete urine collections were made during all periods and were processed generally within 48 hours after collection. Qualitative and quantitative ketosteroid patterns were determined in all periods by the methods of steroid fractionation previously described (1, 2). Briefly, these methods include sequential and separate hydrolysis of steroid conjugates with $\beta$-glucuronidase and mild acid treatment, preparation of neutral fractions from ethereal extracts of the hydrolysates, fractionation of neutral extracts into "ketonic" and "nonketonic," and "$\alpha$-" and "$\beta$-ketosteroid" subfractions, and isolation of individual $\alpha$-ketosteroids by quantitative paper chromatography with steroid identification by infrared spectrometry when necessary.

The proof of monozygosity of the triplets was based on information derived from both fetal membrane examination and the similarity method (3). These triplets were subjects of long-term study by Sontag, Nelson and Reynolds (4, 5) at the Fels Research Institute for the Study of Human Development, Antioch College, Yellow Springs, Ohio, and have been carefully followed from birth. The birth membranes were carefully preserved following delivery and were examined in detail by Sontag and Nelson (4) and G. W. Streeter of the Carnegie Institution of Washington. The principal features of their description are summarized: there was a single placenta, the membranous surface of which was divided into three somewhat unequal parts by two membranous septa. On section, one of these membranes consisted of two amnions fused back to back. The other also showed two amnions but between them was another layer, evidently a much reduced chorionic septum containing remnants of blood vessels. There was no indication of fusion of two placentae. The evidence of the membranes thus indicates the monozygotic, dichorionic origin of these triplets. Similarity in a large number of physical and mental traits was also considered in establishing the single egg origin of the triplets and these have been reviewed by Sontag and Nelson (4). In addition to the physical traits described above, detailed blood typing was performed (through the courtesy of Dr. A. S. Weiner, Brooklyn, N. Y.) at the time of this study and all three subjects had identical blood reactions as follows: O, M, k, $Rh_1$, rh, P and F.

The triplets were born in the following order: F, weight 2.0 kg; J, weight 1.8 kg; and H, weight 1.6 kg. All three were artificially fed after birth. J and F gained well on this schedule but H began to regurgitate his feedings. He gained weight slowly and the nutritional problem persisted with such severity that he was not

* Department of Medicine, The University of Chicago.

† Operated by the University of Chicago for the United States Atomic Energy Commission.

expected to survive during the greater part of the first 6 months following birth. At 14 weeks, he weighed 2.8 kg, compared with 4.1 kg for J and 4.4 kg for F. At 6 months however, the gastrointestinal difficulty of Subject H ceased and he began to gain weight rapidly. At 2 years, H weighed 11.3 kg, F weighed 12.7 kg, and J weighed 12.3 kg; this weight ratio continued into adulthood. All three were raised together in a community of moderate size in Ohio, where they continue to live. Except for the precarious postnatal experience of Subject H, their health has been and continues to be excellent.

## RESULTS

Steroid patterns were studied in each subject during eight of the periods noted above. These were the two pretreatment control periods (*1* and *2*), the three ACTH periods (*3–5*), the postcontrol period (*6*) immediately following the last day of ACTH, and the postcontrol periods on the third day (*8*) and fifth day (*10*) after ACTH. During the second study on Subject H, steroid patterns were determined during one control day and on each of the three days on ACTH. The following abbreviations are used in the figures: A, for androsterone (3$\alpha$-hydroxyandrostane-17-one); E, for etiocholanolone (3$\alpha$-hydroxyetiocholane-17-one); OHA, for 11$\beta$-hydroxyandrosterone (3$\alpha$,11$\beta$-dihydroxyandrostane-17-one); OHE, for 11$\beta$-hydroxyetiocholanolone (3$\alpha$,11$\beta$-dihydroxyetiocholane-17-one), and 11 = OE, for 11-ketoetiocholanolone (3$\alpha$-hydroxyetiocholane-11,17-dione).

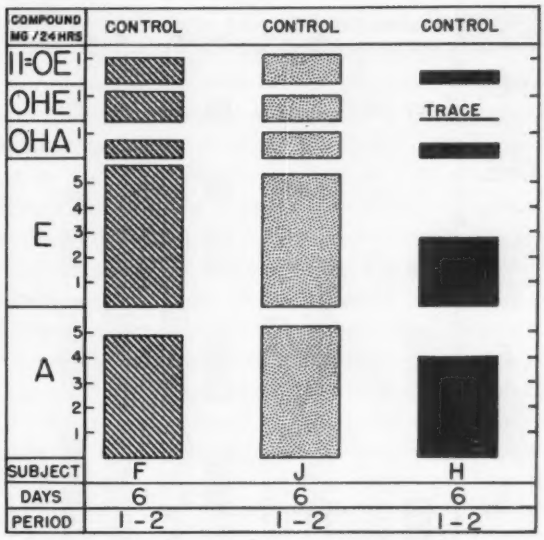



FIG. 1. STEROID PATTERNS IN ADULT MALE DICHORIONIC IDENTICAL TRIPLETS.

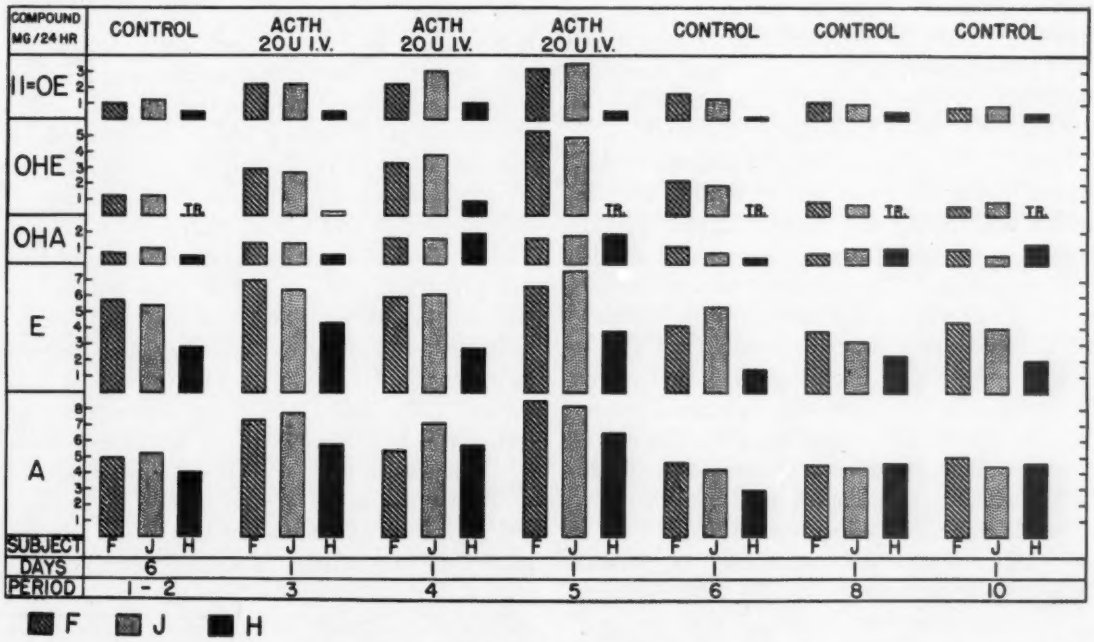



FIG. 2. PRODUCTION OF STEROIDS DURING CONTROL AND ACTH PERIODS BY ADULT MALE MONOZYGOTIC DICHORIONIC TRIPLETS.

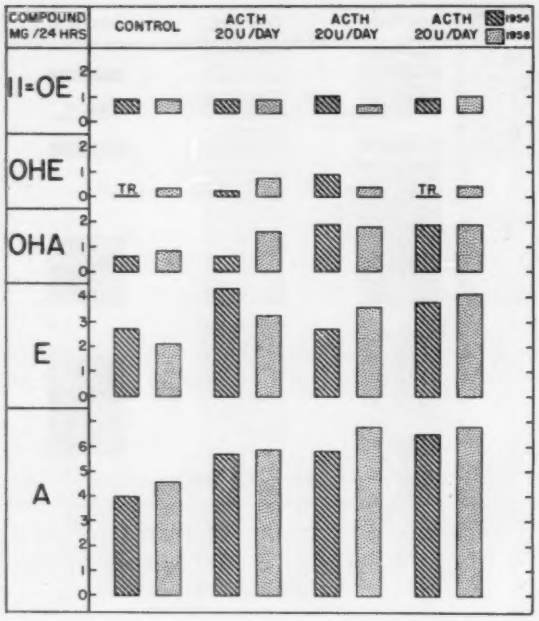



FIG. 3. STEROID PRODUCTION BY SUBJECT H DURING CONTROL AND ACTH PERIODS IN STUDIES MADE FOUR YEARS APART.

Quantitative values for individual ketosteroids during the two combined pretreatment control periods (Periods *1* and *2*) for each triplet are shown in Figure 1. Production of 11-oxygenated and 11-desoxysteroids by F and J was essentially equivalent. Production of all these steroids by H was markedly lower than that of his brothers.

Figure 2 shows the ketosteroid patterns of these triplets during the three days of ACTH administration and during the various control periods. The effect of ACTH was to increase production of 11-desoxy and 11-oxygenated adrenal steroids by J and F in comparable fashion. The decline in steroid production after cessation of ACTH by these two subjects was essentially identical as well. There was a marked difference in adrenal responsiveness to ACTH demonstrated by Subject H. Total production of 11-desoxysteroids during the ACTH periods was considerably less than that of his brothers. The 11-oxysteroids, 11 = OE and OHE, which are metabolites of cortisol, showed slight increase on ACTH administration, but this was in no way comparable with the vigorous rise exhibited by his brothers. Interestingly, OHA, which is the major metabolite of 11$\beta$-hydroxy $\Delta^4$-androstene-3,17-dione (6), increased from a control level slightly less than that of his brothers to a somewhat higher level than that of either F or J during the third day of ACTH.

Figure 3 demonstrates the ketosteroid production of H during control and ACTH periods in an experiment conducted four years after that shown in Figure 2. The striking failure of the 11-oxygenated etiocholane derivatives to increase significantly under ACTH stimulation is once more noted and the excellent quantitative agreement with the previous values is impressive evidence for the validity of the methodology employed.

## DISCUSSION

The patterns of ketosteroid production have been described in detail in previous reports from these laboratories. These steroid patterns differ quantitatively from person to person (1, 2) but are reproducible and constant (7) for a given individual over periods of time. Although subject to alteration by such influences as endocrine (8–10) and nonendocrine disease (11, 12) or by exogenous hormone administration (2, 8, 9, 13, 14) the principal determinants of these patterns under physiological conditions apparently remain well defined. The results of the present study indicate that both genetic and extra-genetic factors play a significant role in determining the characteristics of the steroid pattern in man. The production of 11-desoxy and 11-oxygenated ketosteroids by two of the triplets (F and J) was identical, within experimental error, during all control periods and during each day on ACTH. The metabolites examined originate from at least five precursors; i.e., A and E from testosterone and dehydroisoandrosterone as well as from $\Delta^4$-androstene-3,17-dione (15), the probable "adrenal androgen"; OHE and 11 = OE principally from cortisol (10); and OHA from 11$\beta$-hydroxy $\Delta^4$-androstene-3,17-dione (6). It is evident, therefore, that two of these triplets exhibited a striking similarity, during all of the periods studied, not only in steroid excretion, but also in the rate and pattern of biosynthesis of steroid hormones, the release of these substances into the blood stream, and the enzymatically-determined structural transformations of these hormones to the metabolites isolated and measured. In view of the individuality

of these patterns in normal men (1), the variable adrenal response to ACTH by different men (16), and the established monovular origin of the triplets, the striking similarity of the steroid patterns of F and J in all periods could not be attributed to chance. These data, therefore, may be considered to demonstrate the existence of an important genetic determinant of the characteristic pattern of steroid hormone production and metabolism in man.

There was a significant divergence of the steroid patterns of H from his brothers. Subject H produced smaller amounts of 11-desoxy and 11-oxysteroids during the control period (Figure 1), and during the administration of ACTH his steroid production failed to increase in comparable manner (Figure 2). This difference persisted through the post-ACTH control periods and was likewise demonstrable four years later (Figure 3).

These results with Subject H clearly indicate the existence of an extra-genetic influence operating to modify either the pattern of his steroid hormone production, or steroid hormone metabolism to the compounds isolated, or both. The intrauterine locus of action of this modifying influence is suggested by the small birth weight and subsequent deficient growth pattern of this triplet. The precise nature of this influence cannot be established with certainty, but its selective effect on Subject H, together with information derived from previous twin studies in man (17), permit a plausible explanation. Single egg twins may be either dichorial or monochorial with the latter sharing a mutual fetal circulation from early gestation to birth. This common prenatal circulation operates through a variety of intravascular anastomoses, and is consistently associated with a functional vascular asymmetry resulting in a disproportionate distribution of fetal blood supply to one twin partner. This asymmetry of the mutual circulation of monochorial pairs may be lethal or may result in serious handicap to the intrauterine or neonatal development of the deprived twin. Although there may be considerable postnatal recovery from this early nutritional deficit, the more frequent occurrence of physiological differences between monochorial, as compared with dichorial, single egg twins indicates that many of the effects are lasting (17).

The triplet secundines were dichorial in character (4) indicating a mutual fetal circulation for two of the triplets, comparable with that of monochorial twins. In view of the circulatory imbalance of such a fetal blood supply, it can be presumed that one of this pair of triplets during gestation was consistently deprived of a fetal blood flow equivalent to that of his brothers. The small birth weight and deficient postnatal growth rate of Subject H make it likely that he was the handicapped member; it is suggested that his difference in production or metabolism of steroids represents a physiological aftermath of a relatively deficient fetal blood supply. This same vascular factor may indeed have proved lethal at an early stage to the anticipated fourth embryonic individual.

The nature and physiological implications of the hormonal discordance shown by Subject H are of interest. His steroid pattern is deficient only by comparison with his brothers. Indeed, Subject H compares favorably in terms of steroid production with a series of normal males of comparable age (1). However, his response to adrenal stimulation with ACTH was not only different from that of his brothers but was highly unusual when compared with the response of normal men and women (2, 13, 16). Androsterone, etiocholanolone and 11-hydroxyandrosterone increased, clear evidence that a portion of the adrenal secretion was capable of response to a tropic hormone. There was, however, no significant elevation in the two cortisol metabolites, OHE and 11 = OE, despite the administration of ACTH in a dose and by a route known to produce maximal adrenal stimulation (18).

This apparent impairment in response to ACTH may have the following basis. The inability to increase production of 11-oxygenated etiocholane metabolites during adrenal stimulation may denote a failure to enhance adrenal production of cortisol and may therefore indicate a lack of "adrenal reserve" function. This subject would thus have a superficial physiological resemblance to the patients described by Abu Haydar and colleagues (19).

In contrast to these patients, however, the capacity of Subject H for vigorous physical activity, including war service in Korea, was comparable in all respects with that of his brothers and contradicts the likelihood of latent hypoadrenalism.

Alternatively, the failure to demonstrate increased production of 11-oxygenated etiocholane steroids during ACTH administration may be due to metabolism of cortisol to end products of unusual type, or to the consistent transformation of this hormone to $C_{21}$ metabolites such as "tetrahydrocortisol" or the "cortols" and "cortolones" described by Fukushima and associates (20). These possibilities could not be investigated at the time of this study. If shown to occur, they would in any case only serve to emphasize the difference between Subject H and his brothers and affirm in effect the interplay of genetic and extra-genetic determinants of the patterns of steroid production and metabolism in man.

## SUMMARY

The steroid patterns of normal adult, male, dichorionic, monozygotic triplets were examined in detail during control periods and during adrenocortical stimulation with ACTH. In two of the triplets these patterns were virtually identical before, during, and after ACTH administration. In view of the highly individual nature of these patterns, this is impressive evidence of a genetic influence on steroid hormone production and metabolism in man. The third triplet, however, produced significantly smaller amounts of individual ketosteroids, as compared with his brothers, during all periods. This finding is interpreted as evidence for the existence of an extra-genetic influence by means of which steroid production or metabolism had been modified. Related physiological differences in monochorionic twins have been attributed to demonstrated vascular asymmetry and unequal distribution of the mutual fetal circulation to one partner of the pair. The birth membranes of these triplets were dichorial in character and it is evident that a fetal situation comparable with that seen in monochorionic twins existed for two of the brothers. It is presumed, therefore, that the triplet with divergent steroid patterns was the deprived member of this pair and that his differences in production or metabolism of steroids represent a physiological aftermath of a relatively deficient fetal circulation.

## ACKNOWLEGMENT

We wish to express our appreciation to Dr. L. W. Sontag, Director, The Fels Research Institute for the Study of Human Development, Antioch College, Yellow Springs, Ohio, for his assistance in this study.

## REFERENCES

1. Dobriner, K. Studies in steroid metabolism. XIX. The $\alpha$-ketosteroid excretion pattern in normal males. J. clin. Invest. 1953, **32**, 940.
2. Kappas, A., and Gallagher, T. F. Studies in steroid metabolism. XXVIII. The $\alpha$-ketosteroid excretion pattern in normal females and the response to ACTH. J. clin. Invest. 1955, **34**, 1566.
3. Kallmann, F. J. Twin data in the analysis of mechanisms of inheritance. Amer. J. hum. Genet. 1954, **6**, 157.
4. Sontag, L. W., and Nelson, V. L. A study of identical triplets. Part I. Comparison of the physical and mental traits of a set of monozygotic, dichorionic triplets. J. Hered. 1933, **24**, 473.
5. Sontag, L. W., and Reynolds, E. L. Ossification sequences in identical triplets. A longitudinal study of resemblances and differences in the ossification patterns of a set of monozygotic triplets. J. Hered. 1944, **35**, 57.
6. Bradlow, H. L., and Gallagher, T. F. Metabolism of 11$\beta$-hydroxy $\Delta^4$-androstene-3,17-dione in man. J. biol. Chem. 1957, **229**, 505.
7. Dobriner, K. Studies in steroid metabolism. XX. The reproducibility of the urinary steroid pattern in humans. J. clin. Invest. 1953, **32**, 950.
8. Kappas, A., Pearson, O. H., West, C. D., and Gallagher, T. F. A study of "idiopathic" hirsutism; a transitional adrenal abnormality. J. clin. Endocr. 1956, **16**, 517.
9. Gallagher, T. F., Kappas, A., Hellman, L., Lipsett, M. B., Pearson, O. H., and West, C. D. Adrenocortical hyperfunction in "idiopathic" hirsutism and the Stein-Leventhal syndrome. J. clin. Invest. 1958, **37**, 794.
10. Gallagher, T. F. On alterations in adrenal function, especially with adrenocortical carcinoma. Cancer Res. 1957, **17**, 520.
11. Dobriner, K., Kappas, A., and Gallagher, T. F. Studies in steroid metabolism. XXVI. Steroid isolation studies in human leukemia. J. clin. Invest. 1954, **33**, 1481.
12. Kappas, A., Milhorat, A. T., Rhoads, C. P., and Gallagher, T. F. A study of adrenocortical and testicular physiology in dystrophia myotonica. A preliminary report. Amer. J. phys. Med. 1955, **34**, 303.
13. Kappas, A., Dobriner, K., and Gallagher, T. F. Studies in steroid metabolism. XXVII. A comparison of the steroid response to ACTH and cortisone in normal young men. J. clin. Invest. 1955, **34**, 1559.
14. Hellman, L., Bradlow, H. L., Zumoff, B., Fukushima, D. K., and Gallagher, T. F. Thyroid-androgen interrelations and the hypocholesterolemic effect of androsterone. J. clin. Endocr. 1959, **19**, 936.

15. Dorfman, R. I., and Shipley, R. A. Androgens: Biochemistry, Physiology, and Clinical Significance. New York, John Wiley and Sons, 1956.
16. Dobriner, K. Studies in steroid metabolism. XXII. The adrenal response to ACTH in young and old men. J. clin. Invest. 1954, **33**, 222.
17. Price, B. Primary biases in twin studies. A review of prenatal and natal difference-producing factors in monozygotic pairs. Amer. J. hum. Genet. 1950, **2**, 293.
18. Renold, A. E., Jenkins, D., Forsham, P. H., and Thorn, G. W. The use of intravenous ACTH: A study in quantitative adrenocortical stimulation. J. clin. Endocr. 1952, **12**, 763.
19. Abu Haydar, N. A., St. Marc, J. R., Reddy, W. J., Laidlaw, J. C., and Thorn, G. W. Adrenocortical insufficiency with normal basal levels of urinary 17-hydroxycorticoids: Diagnostic implications. J. clin. Endocr. 1958, **18**, 121.
20. Fukushima, D. K., Leeds, N. S., Bradlow, H. L., Kritchevsky, T. H., Stokem, M. B., and Gallagher, T. F. The characterization of four new metabolites of adrenocortical hormones. J. biol. Chem. 1955, **212**, 449.

# ESTIMATION OF THE CONSTANCY OF DENSITY OF THE FAT-FREE BODY *

By EDWARD J. WERDEIN † AND LAURENCE H. KYLE

*(From the Department of Medicine, Georgetown University School of Medicine, the Georgetown University Hospital, Washington, D. C., and St. Mary's Hospital, Rochester, N. Y.)*

(Submitted for publication October 15, 1959; accepted December 4, 1959)

Implicit in the estimation of body fat by densitometric measurement is the assumption that the fat-free body mass of normal persons possesses a constant density. This is true whether one utilizes the concept of Behnke, Feen and Welham (1), who visualized a lean body mass of specific gravity 1.1, containing a small amount of essential lipids, or the standard man of Keys and Brôzek (2), who is neither starved nor obese. Estimation of body fat by determination of total body water is, as pointed out by Messinger and Steele (3), subject to the same basic assumption. The formula of Siri (4), developed to avoid the assumption of a constant water content of the fat-free body and to permit correction for abnormal hydration, is nonetheless predicated on the basis of a known and constant density of the nonfat solids of the body.

Unusual deviations in the density of the fat-free body are to be expected in conditions of abnormal hydration of the body and with significant loss or gain of protein and minerals. Consideration must also be given to the possibility that there exists considerable deviation of density of the fat-free body in normal subjects, if for no other reason than the known biological variability in other spheres. Such deviation would not necessarily involve hydration but could be the result of variation of density of the mineral mass of the body or deviation from the estimated mineral: protein ratio.

Although no direct approach is available, indirect methods can provide some evidence as to the variability of fat-free body density. Significant deviation of percentage of water in the fat-free body of normally hydrated subjects, estimated by parallel measurements of body density and total body water, would indicate abnormal density of the fat-free body. Such study was conducted in a group of subjects of varying fat content and in patients selected because of known abnormality of their mineral mass.

## MATERIALS AND METHODS

Subjects of the study were normal hospital personnel and selected patients with a wide range of body build who were considered to be "normal" as far as body constituents and compartments were concerned. Because of the facilities for underwater weighing, the subjects were predominately male and with two exceptions, noted in Table I, ranged in age from 18 to 39. To highlight deviation from the normal on the basis of abnormal skeletal density, measurements were also made in osteoporosis and in a man with osteosclerosis (marble bone disease).

Body density (D) was measured by underwater weighing, with correction for residual lung volume by the helium dilution technique, as described from this laboratory (5). Total body water (TBW) was measured by volume distribution of antipyrine or $D_2O$ as outlined previously (5). Radio-antipyrine was also used in a few subjects as described by Talso and associates (6). Body fat (F) was estimated by substitution into the Pace-Rathbun equation (2), corrected for temperature and density of fat:

$$\% \text{ fat} = \frac{5.120}{D} - 4.684. \qquad (A)$$

Weight of the fat-free body (FFB) was determined by subtracting the amount of fat from the total body weight. Hence, the water content of the fat-free body was calculated:

$$\% \text{ water} = \frac{TBW}{FFB}$$

and used as an index of the degree of deviation of the density of the fat-free body from the norm, not with the idea that there existed abnormality of hydration, but with the assumption that abnormally high or low percentage of water would reflect variability of the fat-free body density.

* This study was supported by a contract between Georgetown University and the Office of Naval Research. Reproduction in whole or in part is permitted for any purpose of the United States Government.

† Former Research Fellow of the National Institutes of Health. Present address: St. Mary's Hospital, Rochester, N. Y.

The measured values for D and TBW were substituted into the fat prediction equation of Siri (4) to minimize any possible error in fat estimation introduced by a true abnormality of hydration:

$$\% F = \left(\frac{2.118}{D} - 0.780\ TBW - 1.354\right) \times 100. \quad (B)$$

The density of the fat-free body ($D_{ffb}$) was obtained by dividing fat-free body weight by its volume.

## RESULTS

Table I summarizes the results of the outlined determinations in 30 normal subjects. Body density ranged from 0.962 in an extremely obese woman to 1.079 in a thin, well-conditioned man. Percentage of water in the fat-free body, as determined by independent measurements of body density and total body water (A) was 67.5 ± 5.5 but the range extended from 53.1 to 74.8 per cent. Calculations based on substitution of D and TBW in Siri's fat prediction equation demonstrated, as would be expected by the basic assumptions of the equation, a higher mean value and less spread of percentage of water in the fat-free body.

TABLE I

*Relationship between total body water and fat-free body* *

| Subject | Weight | D | TBW | $H_2O$/FFB (A)† | $H_2O$/FFB (B)‡ | Sex | Age |
|---|---|---|---|---|---|---|---|
| | *kg* | | | % | % | | *yrs* |
| 1 | 78.8 | 1.059 | 35.5 | 53.1 | 63.8 | M | 28 |
| 2 | 75.9 | 1.064 | 37.0 | 55.9 | 65.6 | M | 26 |
| 3 | 81.0 | 1.049 | 36.6 | 56.3 | 65.7 | M | 24 |
| 4 | 76.1 | 1.060 | 39.2 | 60.3 | 67.9 | M | 27 |
| 5 | 85.5 | 1.037 | 40.2 | 62.9 | 69.2 | M | 28 |
| 6 | 85.0 | 1.038 | 40.5 | 63.5 | 69.6 | M | 30 |
| 7 | 93.1 | 1.019 | 39.5 | 64.3 | 69.9 | M | 45 |
| 8 | 79.5 | 1.044 | 40.1 | 64.7 | 70.2 | M | 32 |
| 9 | 66.1 | 1.064 | 37.5 | 65.1 | 70.5 | M | 28 |
| 10 | 72.9 | 1.079 | 45.1 | 65.8 | 70.8 | M | 27 |
| 11 | 71.5 | 1.037 | 35.9 | 67.2 | 70.7 | M | 31 |
| 12 | 63.4 | 1.046 | 33.8 | 67.6 | 71.6 | M | 35 |
| 13 | 74.3 | 1.056 | 42.1 | 67.8 | 71.7 | M | 29 |
| 14 | 70.8 | 1.017 | 31.5 | 68.5 | 71.9 | F | 28 |
| 15 | 179.5 | 0.995 | 66.2 | 68.5 | 73.4 | M | 28 |
| 16 | 63.2 | 1.062 | 37.6 | 69.0 | 72.2 | M | 30 |
| 17 | 83.7 | 1.057 | 48.6 | 69.1 | 72.3 | M | 26 |
| 18 | 74.9 | 1.046 | 41.0 | 69.4 | 72.4 | M | 33 |
| 19 | 121.4 | 0.962 | 30.5 | 69.5 | 72.3 | F | 18 |
| 20 | 73.0 | 1.043 | 39.5 | 69.8 | 72.6 | M | 29 |
| 21 | 81.9 | 1.050 | 46.2 | 69.8 | 72.6 | M | 27 |
| 22 | 171.5 | 0.976 | 52.4 | 69.8 | 72.8 | M | 24 |
| 23 | 65.5 | 1.041 | 36.2 | 72.1 | 73.7 | F | 23 |
| 24 | 66.0 | 1.009 | 29.3 | 72.7 | 73.8 | F | 39 |
| 25 | 59.2 | 1.051 | 35.0 | 72.8 | 73.8 | M | 27 |
| 26 | 77.1 | 1.048 | 45.1 | 73.2 | 74.2 | M | 33 |
| 27 | 56.8 | 0.979 | 19.0 | 73.6 | 73.9 | F | 14 |
| 28 | 50.4 | 1.046 | 29.3 | 73.6 | 74.4 | F | 21 |
| 29 | 129.4 | 1.028 | 68.0 | 74.7 | 74.6 | M | 20 |
| 30 | 72.3 | 1.070 | 48.6 | 74.8 | 74.8 | M | 31 |
| Mean | | | | 67.5 | 71.4 | | |
| SD | | | | ±5.5 | ±2.7 | | |

* D = density, TBW = total body weight; FFB = fat-free body.
† Modified Pace-Rathbun equation.
‡ Siri formula; see Methods for these equations.

TABLE II

*Reproducibility of body partition studies*

| Case | Weight | D | TBW | $H_2O$/FFB (A) | $H_2O$/FFB (B) | * Antipyrine space | $I^{131}$ A-P space | $D_2O$ space |
|---|---|---|---|---|---|---|---|---|
| | *kg* | | | % | % | | | |
| 31 | 62.3 | 1.051 | 36.7 | 72.5 | 73.8 | | | |
| | 63.1 | 1.046 | 36.0 | 72.3 | 73.5 | | | |
| | 70.3 | 1.042 | 36.2 | 66.9 | 71.3 | | | |
| | 66.8 | 1.043 | 34.7 | 67.0 | 71.4 | | | |
| | 69.3 | 1.035 | 36.0 | 70.5 | 72.7 | | | |
| 3 | 81.0 | 1.049 | 36.6* | 56.3 | 65.7 | 36.6 | 36.6 | 37.8 |
| | 81.3 | 1.045 | 36.0* | 56.6 | 65.9 | 36.0 | 37.2 | 37.5 |
| 10 | 72.9 | 1.079 | 45.1 | 65.8 | 70.8 | | | |
| | 74.1 | 1.080 | 45.6 | 65.2 | 70.4 | | | |

Table II summarizes repeated studies conducted in three subjects. Case 31 (not shown in Table I) was measured five times over a period of 15 months, at various stages of nutrition. Although there was change in body density, attributable largely to fluctuation in body fat, there was minimal change in percentage of water of the fat-free body. The variations noted are consistent with the expected error of the methods and stand in sharp contrast to the much greater variation among the entire group of normal subjects.

Case 3 (Table II) summarizes two measurements, one year apart, of density and TBW in a healthy subject demonstrating an extremely high density of the fat-free body. Because the latter was so far above "normal," careful recheck was made of body density (underwater weighing), and all three methods—antipyrine, $I^{131}$-antipyrine, and $D_2O$—were utilized to determine total body water.

Case 10 (Table II) had the highest body density of the normal subjects. Replicate determinations of D and TBW showed again a lesser degree of variation in percentage of water and density of

TABLE III

*Effect of altered mineral mass upon total body water and fat-free body*

| | Weight | D | TBW | $H_2O$/FFB (A) | $H_2O$/FFB (B) | $D_{ffb}$* |
|---|---|---|---|---|---|---|
| | *kg* | | | % | % | |
| Osteosclerosis | 48.6 | 1.114 | 21.1 | † | 55.0 | 1.189 |
| Osteoporosis | 49.1 | 1.008 | 29.4 | 99.0 | 83.1 | 1.057 |
| Average "normals" | | | | 67.5 | 71.4 | |

* $D_{ffb}$ = density, of fat-free body.
† Body fat had a negative value.

the fat-free body than was noted among the group of normal subjects.

The profound effects of pathological degrees of increased or decreased bone density on *in vivo* dissection by densitometric or volume distribution techniques are illustrated in Table III. Measurements of the type outlined above in a subject with osteosclerosis and one with osteoporosis are compared with the mean value of the normal subjects. The very low percentage of water in osteosclerosis and the high value in osteoporosis reflect the significant role of bone density in the estimation of total body fat. When calculations are extended to determine density of the fat-free body, a marked deviation from the norm is noted. These striking abnormalities are, however, merely the extremes of a broad spectrum seen in the normal subjects.

## DISCUSSION

Keys and Brôzek (2) have pointed out that the density of the fat-free body is unknown. Short of dissection of a large number of human bodies after *in vivo* analysis, it is unknowable. Furthermore, because adipose tissue contains both water and protein in addition to its larger component of pure fat, density of the lean body could deviate from any selected norm with overfeeding or starvation.

Data of the type reported here, bearing on body constituents, have been furnished by Messinger and Steele (3) who measured 9 normal subjects, and by Osserman, Pitts, Welham and Behnke (7) who studied 81 normal servicemen. Both of these groups reported mean values for percentage of water in the FFB consistent with the predictions of McCance and Widdowson (8) and the animal studies of Rathbun and Pace (9). There was, however, considerable range even in these relatively homogeneous groups and the even greater range in our subjects could be attributed to several factors. These include systematic error, true abnormality of body water, and wide variation of the density of the dry fat-free solids. The minimal methodological error previously reported from this laboratory (10), the error of both D and TBW reported by others, and the similarity of replicate determinations in the subjects shown in Table II attest to the fact that the wide range of values must involve factors other than methodological error. Data derived from both *in vivo* measurement and direct tissue analysis indicate that major deviation of percentage of water in the FFB is to be expected only with profound abnormality of hydration. Consequently, it would appear that the range of variability noted in this study must be attributed to a not inconsiderable range of density of the dry fat-free solids. This probability is supported by the even greater variability demonstrated in the patients with known skeletal abnormality. Considering the biological variation of human subjects in other spheres, it would be surprising to expect a constant weight per volume ratio of the fat-free body, particularly in view of the fact that an individual's supporting tissue must reflect that which it supports and the stresses to which it must become accustomed. Although muscle mass might vary to some degree, and thus offer partial explanation for deviation of density of the fat-free body, the much greater density of mineral mass would render this body constituent a more significant determinant of lean tissue density.

It is apparent that any disorder that affects skeletal density would prohibit accurate fat estimation by either densitometric or volume distribution techniques, both of which depend upon a constant density of the fat-free body or a constant mineral-protein ratio. With realization that material loss of minerals may occur before there exists radiological indication of such decrease in bone density, it seems logical that fat estimation from densitometric or volume distribution methods could be erroneous in older patients. Decrease in bone density, as would occur in mild osteoporosis, could affect the standard of reference to such

a degree that an overestimation of body fat could be expected in patients over 40.

It must be pointed out, however, that much less error would accrue from serial determinations conducted to estimate change in total body fat (11). Because of the slow mineral turnover and minimal effect of protein change on body density, satisfactory estimation of loss or gain in fat is feasible even though the original estimation of total fat is in error. As pointed out by Siri, and by Keys and Brôzek, concern regarding a constant density of the fat-free body should serve to stimulate development of methods for measurement of body fat independent of those outlined above. While *change* in fat is more accurately estimated by densitometric techniques, the independent method of coupling TBW with balance study appears suitable (11). Densitometric and volume distribution methods have stimulated interest in the lipid component of the body, but precise assay of body fat remains dependent upon truly independent methods of quantitative analysis.

## SUMMARY

The *in vivo* estimation of total body fat has provided useful information, particularly in young healthy males. When current techniques were applied to a heterogeneous group of subjects with apparently normal hydration, but who had varying degrees of physical fitness and obesity and who varied in age, the inherent weakness of our present techniques and some of their basic assumptions become apparent. Such discrepancies are highlighted in subjects with decreased or increased density of the skeleton. While useful for the estimation of *change* in total body fat, densitometric and volume distribution methods possess the inherent weakness of dependency upon the assumption that the fat-free body in normally hydrated individuals demonstrates a constant density. This study indicates that fat-free body density varies considerably among normal individuals and to an extreme degree in patients with bone disease.

## ACKNOWLEDGMENT

We are grateful for the assistance of Drs. Richard J. Meyer and John J. Canary in the conduct of this study, and to the personnel of the Experimental Diving Unit, Naval Gun Factory, for the performance of underwater weighing.

## REFERENCES

1. Behnke, A. R., Jr., Feen, B. G., and Welham, W. C. The specific gravity of healthy men. Body weight divided by volume as an index of obesity. J. Amer. med. Ass. 1942, **118**, 495.
2. Keys, A., and Brôzek, J. Body fat in adult man. Physiol. Rev. 1953, **33**, 245.
3. Messinger, W. J., and Steele, J. M. Relationship of body specific gravity to body fat water content. Proc. Soc. exp. Biol. (N. Y.) 1949, **70**, 316.
4. Siri, W. E. Body composition from fluid spaces and density: Analysis of methods. Donner Laboratory of Biophysics and Medical Physics, Univ. of Calif., Berkeley, Calif. UCRL, 1956, March, 3349.
5. Kyle, L. H., Meyer, R. J., Schaaf, M., and Werdein, E. J. The effects of Compound A 11-dihydrocorticosterone and Compound F 17-hydrocorticosterone on total body fat. J. clin. Invest. 1956, **35**, 1045.
6. Talso, P. J., Lahr, T. N., Spafford, N., Ferenzi, G., and Jackson, H. R. O. A comparison of the volume of distribution of antipyrine, N-acetyl-4-amino-antipyrine and I-131-labeled 4-iodo-antipyrine in human beings. J. Lab. clin. Med. 1955, **46**, 619.
7. Osserman, E. F., Pitts, G. C., Welham, W. C., and Behnke, A. R. In vivo measurement of body fat and body water in a group of normal men. J. appl. Physiol. 1950, **2**, 633.
8. McCance, R. A., and Widdowson, E. M. A method of breaking down the body weights of living persons into terms of extracellular fluid, cell mass and fat, and some applications of it to physiology and medicine. Proc. roy. Soc. B 1951, **138**, 115.
9. Rathbun, E. N., and Pace, N. Studies on body composition. I. The determination of total body fat by means of the body specific gravity. J. biol. Chem. 1945, **158**, 667.
10. Kyle, L. H., Welham, W. C., Doolan, P. D., and Schaaf, M. Study of the extrathyroidal effects of thyroid-stimulating hormone. J. clin. Endocr. 1954, **14**, 1029.
11. Kyle, L. H., Werdein, E. J., Canary, J. J., and Pachuta, B. Measurement of change in total body fat. J. clin. Invest. 1959, **38**, 1475.

# THE EFFECT OF NEUROHYPOPHYSEAL HORMONES ON THE PERMEABILITY OF THE TOAD BLADDER TO UREA*

By ROY H. MAFFLY,† RICHARD M. HAYS,‡ EZRA LAMDIN § AND ALEXANDER LEAF ‖

*(From the Departments of Medicine, Massachusetts General Hospital and the Harvard Medical School, Boston, Mass., and the Howard Hughes Medical Institute)*

(Submitted for publication October 27, 1959; accepted November 20, 1959)

With the introduction of the countercurrent hypothesis (1) to explain renal concentrating ability there has been renewed interest in the role of urea in renal tubular function. Recent studies (2–5) suggest that urea makes a major contribution to the urinary concentrating mechanism. If this is the case, one might expect important differences in the permeability of the renal tubule to urea in the presence or absence of antidiuretic hormone. Further, such movement of urea across the tubule might be passive, or effected by means of an active transport system, such as has been described for the bull frog kidney (6–8).

The difficulty of exploring such problems in the intact mammalian kidney need not be emphasized. It would be desirable to use a simpler system, in which the movement of urea across living cells could be measured directly. The studies of Ussing, Zerahn, Koefoed-Johnsen, and Andersen (9–12) have demonstrated the usefulness of isolated, surviving amphibian membranes as models for the study of active transport and other membrane phenomena. In the studies reported below we have used the toad bladder, a tissue that resembles the mammalian renal tubule in several respects (13).

Our results indicate that: *1*) the permeability of the toad bladder to urea is low but increases strikingly in the presence of mammalian neurohypophyseal hormones; *2*) there is no evidence for active transport of urea across the toad bladder; *3*) the enhanced diffusion of urea following hormone appears to be a result primarily of the increased permeability of the mucosal surface of the membrane; *4*) although urea movement is passive, the membrane exhibits a high degree of selectivity in that the diffusion of compounds structurally similar to urea may not be affected by neurohypophyseal hormones.

## METHODS AND MATERIALS

Studies were carried out with the toad, *Bufo marinus*. The animals were used throughout the year, being kept on moist earth at room temperature and force-fed with meal worms once or twice weekly. A previous publication from this laboratory (13) outlines the general techniques employed, and describes the histological appearance of the toad bladder, its electrical properties, and its active transport of sodium ion from the mucosal to the serosal surface which is characteristically stimulated by neurohypophyseal hormones.

In the present studies, the experimental protocol generally observed was as follows: after doubly pithing the toad, one-half of the bilobed urinary bladder was removed through a ventral abdominal incision, rinsed in frog Ringer solution, and mounted as a diaphragm separating two halves of a lucite chamber similar in design to that described by Ussing and Zerahn (9). Equal volumes (usually 10 ml) of Ringer solution bathed each surface of the membrane. In the studies with urea, 0.1 mg per ml each of penicillin G and streptomycin sulfate was added to the bathing media to prevent bacterial decomposition of urea, and in most instances sufficient unlabeled urea was added to raise the concentration in the media to 2 mmoles. The spontaneous membrane potential was brought to zero by the short-circuiting technique of Ussing and Zerahn (9). A tracer amount of the radioactive substance to be studied was then added to one

* Supported by grants from the John A. Hartford Foundation, Inc., the United States Public Health Service (Grant H-2822 of the National Heart Institute), and the American Heart Association, Inc.

† United States Public Health Service Postdoctorate Research Fellow 1958–1960. Present address: Cardiovascular Research Institute, University of California Medical Center, San Francisco, Calif.

‡ Postdoctorate Fellow of the American College of Physicians 1958–1959 and currently Postdoctorate Research Fellow of the American Heart Association, Inc.

§ Clinical Investigator, Veterans Administration. Present address: Boston Veterans Administration Hospital, Boston, Mass.

‖ Investigator, Howard Hughes Medical Institute.

side of the bladder and its rate of appearance on the other side was determined. From this a permeability coefficient, $K_{trans}$, could be obtained which is defined as the amount of the given substance crossing 1 sq cm of membrane surface per second under a driving force of unit concentration gradient; thus

$$K_{trans} = \frac{\text{increase in counts on the unlabeled side}}{\text{concentration of counts on labeled side} \times \text{area of membrane} \times \text{time}},$$

and has the dimensions of centimeters per second. In describing the statistics of any series of measurements, (SE) will be used to designate standard error of the mean and (SD) the standard deviation.

In general, control observations were made for two half hour periods, after which neurohypophyseal hormone was added and measurements continued for two further periods. All experiments were carried out well within the period of viability of the tissue which, as judged by relative constancy of the short-circuit current, is at least 6 hours.

In the studies designed to evaluate the possibility of active transport of urea, double labeling experiments were carried out with $N^{15}$- and $C^{14}$-labeled urea added to opposite bathing media; 50 to 200 mg of $N^{15}$-urea was used per study (final total urea concentration of 40 to 330 mmoles) and a corresponding amount of unlabeled compound was therefore added to the opposite side together with the tracer amounts of $C^{14}$-urea in order to equalize the concentrations of urea bathing the two surfaces of the membrane. In this group of studies, neurohypophyseal hormones were either not added or else were introduced at the beginning of the experiment.

To assess a possible role of carrier-mediated transport of urea, vasopressin was administered to the short-circuited bladder and the permeability to $C^{14}$-urea added to the mucosal side was measured. Following an initial half hour control period, sufficient unlabeled urea was added to the mucosal medium to bring the total concentration of urea to 25 or 50 mmoles, and measurements were continued for a second and third half hour period.

To determine the amount of labeling of tissue water by urea, paired halves of the same bladder were mounted in two separate chambers and permeability studies were carried out as usual, except that $C^{14}$-urea was added to the mucosal side only, and vasopressin was administered to only one of the paired halves. At the end of the experiment, the bathing solutions were removed and the tissues carefully blotted on Whatman no. 54 filter paper in such a way as to avoid contamination of the serosal surface by the isotope-containing mucosal fluid. The tissue was quickly weighed (B-6 semi-micro balance, Mettler Instrument Corp., Hightstown, N. J.) and then homogenized with 0.25 ml of 10 per cent trichloroacetic acid; the homogenate was diluted with 3.0 ml of Ringer's solution and then centrifuged for 20 minutes at approximately 4,000 rpm. The activity of $C^{14}$ in the supernatant fluid was determined. The water content of the blotted toad bladder, considered to be equal to the loss in weight occurring after drying at 95° C for 24 hours, averaged $80.8 \pm 1$ per cent (SD) in 28 measurements,[1] and hence a figure of 81 per cent was utilized to calculate the concentration of labeled urea in the total tissue water. The concentration of $C^{14}$-urea in tissue water divided by its concentration in the bathing medium, times 100, has been termed "per cent labeling."

The neurohypophyseal material used was commercial vasopressin (Pitressin, Parke, Davis and Co., Detroit, Mich.) 0.05 to 0.2 unit per ml of bathing medium and, in a few experiments, purified oxytocin (0.02 unit per ml) and arginine-vasopressin (0.0085 unit per ml), the latter kindly furnished by Dr. Vincent du Vigneaud. The effects of neurohypophyseal preparations tested were qualitatively similar and the term "hormone" will be used subsequently to refer to these preparations. Isotopes used were urea-$C^{14}$, acetamide-1-$C^{14}$, thiourea-$S^{35}$, and water-$H^3$ (New England Nuclear Corp., Boston, Mass.); urea-$N^{15}$ (Isomet Corp., Palisades Park, N. J. and Abbott Laboratories, North Chicago, Ill.); thiourea-$C^{14}$ (Nuclear-Chicago Corp., Chicago, Ill. and New England Nuclear Corp.); L-arginine-$C^{14}$, choline-methyl-$C^{14}$, glycerol-1-$C^{14}$, glycine-1-$C^{14}$, thiocyanate-$S^{35}$, sucrose-$C^{14}$ (uniformly labeled), and DL-lactic-1-$C^{14}$ acid (Nuclear-Chicago Corp.); inulin carboxylic acid-$C^{14}$ (National Bureau of Standards, Washington, D. C.); tritiated 1,5-anhydroglusitol (courtesy of Dr. Robert K. Crane); $Na^{24}$, $K^{42}$ and $Cl^{35}$ (Atomic Energy Commission, Oak Ridge, Tenn.); and $I^{131}$, $S^{35}O_4$ and colloidal gold, $Au^{198}$ (Abbott Laboratories).

Routine counting of radioactive isotopes was performed with a gas-flow, thin-window counter (automatic sample changer, model 750, Baird-Atomic Instrument, Inc., Cambridge, Mass.). Tritium and a few $C^{14}$ samples were counted with a Tri-carb liquid scintillation spectrometer (Packard Instruments Co., La Grange, Ill.). Great care was taken to keep self-absorption constant when using the gas-flow counter.

$N^{15}$-urea was analyzed as $N^{15}$ gas in a mass spectrometer (model 21-201, Consolidated Electrodynamics Corp., Pasadena, Calif.) after preparation by the following modification of the method of Sprinson and Rittenberg (14): 0.5 or 1.0 ml of medium containing $N^{15}$-urea was diluted in a solution of $N^{14}$-urea to achieve a ratio of $N^{15}$:$N^{14}$ of 0.5 to 3.0 per cent. One ml of this solution was placed in the outer well of a Conway dish containing 0.07 N $H_2SO_4$ in the center well. Urease was added to

[1] This value agreed satisfactorily with the volume of distribution of tritiated water, which in 4 measurements was found to average 78 per cent.

TABLE I

*Permeability of toad bladder to urea*

| Neurohypophyseal hormone | Number of periods | Mean $K_{trans}$ ($\times 10^{-7}$ cm/sec) | Standard error of mean ($\times 10^{-7}$ cm/sec) |
|---|---|---|---|
| Absent | 74 | 26 | 2.5 |
| Present | 41 | 274 | 3.8 |

hydrolyze the urea to ammonia which was then liberated by potassium carbonate and trapped in the center well as ammonium sulfate. The ammonium sulfate was subsequently treated by the procedure of Sprinson and Rittenberg (14). Appropriate standards were run with each group of samples; the calibration curves so obtained proved to be linear over the range employed.

## RESULTS

*Permeability to urea.* The permeability coefficient of the toad bladder for urea (Table I) in the absence of hormone, measured in 74 half hour periods carried out in either direction across the membrane, averaged $26 \pm 2.5$ (SE) $\times 10^{-7}$ cm per second. The range of individual values was large, 1.4 to 91 $\times 10^{-7}$ cm per second.

Upon the addition of hormone to the serosal bathing medium, the permeability rapidly and regularly rose by fivefold to over 100-fold, reaching a mean value of $274 \pm 3.8$ (SE) $\times 10^{-7}$ cm per second with a range of 165 to 479 $\times 10^{-7}$ cm per second. A typical response is recorded in Figure 1. No response was noted when hormone was added to the medium bathing the mucosal surface, as is also the case for active sodium transport (13).

*Specificity of hormonal effect.* The effect of hormone on the permeability of the bladder to urea and to other substances is shown in Table II. The bulk of these substances penetrates the bladder at a relatively low rate which is unaffected by hormone. Water, sodium (in the direction of active transport) and urea show an increased penetration of the membrane following hormone. The permeability to water increases approximately twofold, and to sodium two- to threefold. Urea is distinctive by virtue of its relatively low penetration in the absence of hormone and the large augmentation in permeability in the presence of this hormone.

Two compounds, chosen because of their apparent structural similarity to urea have also been tested (see Figure 1). Acetamide, in which one of the amino groups of urea has been replaced by a methyl radical, behaves in a manner very similar to urea both before and after hormone; whereas thiourea, with a sulfur atom replacing the double bonded oxygen of urea, moves in the same permeability range as urea before vasopressin but shows no response whatsoever to the addition of the hormone.

The following facts lend assurance that the striking difference in behavior of urea and thiourea is valid. *1*) The marked increase in radioactivity which appeared on the opposite side of the bladder after hormone in an experiment with $C^{14}$-urea disappeared upon treatment with urease, indicating that the substance that traversed the membrane was indeed urea. *2*) Identical values for the flux of thiourea were obtained with two different tracers, $C^{14}$- and $S^{35}$-labeled thiourea, respectively. Both of these compounds were chromatographed on paper using a solvent system of butanol 86 per

TABLE II

*Permeability of toad bladder without and with vasopressin*

| Substance | | | $K_{trans} \times 10^{-7}$ cm/sec | |
|---|---|---|---|---|
| | | | Without vasopressin | With vasopressin |
| Colloidal gold | | | 0 | 0 |
| Thiourea<br>Glycerol<br>1,5-Anhydroglusitol<br>Sucrose<br>Inulin | Sodium (passive)<br>Potassium<br>Choline<br>Chloride<br>Iodide | Thiocyanate<br>Sulfate<br>Lactate<br>Glycine<br>Arginine | 1–20 | 1–20 |
| Sodium (active) | | | 35 | 100 |
| Urea | | | 26 | 274 |
| Water | | | 900 | 1,600 |

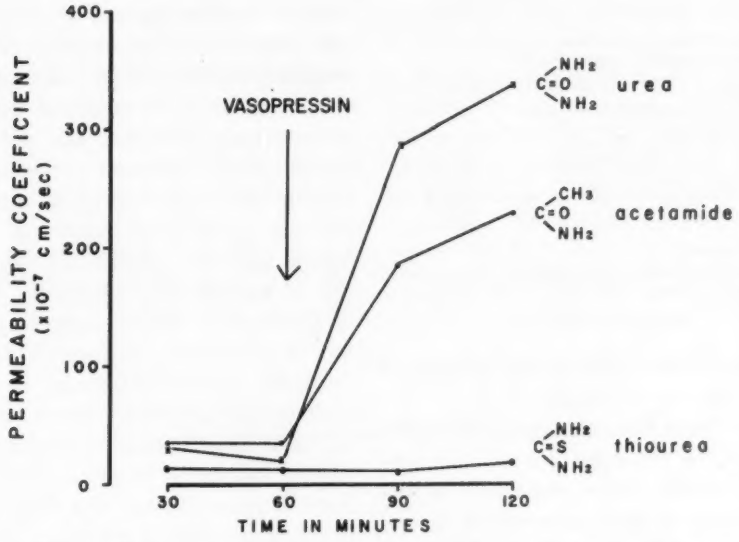



FIG. 1. EFFECT OF NEUROHYPOPHYSEAL HORMONE ON PERMEABILITY OF TOAD BLADDER TO UREA, ACETAMIDE AND THIOUREA. The appropriately labeled molecule was added to the medium bathing one surface of the membrane and its rate of appearance on the opposite side was determined. After two periods of 30 minutes each, vasopressin was added to the serosal medium and an additional two periods determined. In every instance the permeability to urea and acetamide was enhanced by hormone, while permeability to thiourea was unaffected.

cent and water 14 per cent, and both gave single peaks of radioactivity with $R_f$ values of 0.34 to 0.36. *3*) The failure of a hormonal response could not be attributed to inactivation of the hormone by the small amounts of thiourea employed, because in all experiments the usual marked effect of the hormone on the active sodium transport occurred. The hormonal enhancement of urea penetration was also demonstrated in the presence of 1 mmole thiourea in the bathing media.

To determine if the vasopressin effect on urea permeability was contingent upon the presence of sodium transport, urea flux measurements were carried out in the absence of sodium ion by replacing all sodium in frog Ringer with choline. It was found that urea permeability was still markedly enhanced following the hormone although, in the few (four) experiments performed, perhaps not to the same extent as in the presence of sodium.

*The mechanism of penetration.* Ussing (10) has stated the criterion for determining whether the passage of an ion or molecule through a membrane involves its active transport. If the permeability of the membrane for the substance tested is found equal in both directions in the absence of an electrochemical potential gradient for that substance across the membrane and in the absence of net movements of solvent through the membrane, it can reasonably be concluded that active transport of that substance is not taking place.[2]

Such a study was carried out employing $C^{14}$-urea to determine the permeability in one direction and $N^{15}$-urea to determine simultaneously the permeability in the other direction. The method has been described in the preceding section. Four experiments, comprising a total of 17 half hour periods, were performed in the absence of hormone. Three experiments, totaling 13 periods, were performed following the introduction of 2 units of vasopressin to both the mucosal and serosal bathing media. The results are shown in Table III. It can be seen that both before and after hormone

[2] Even with these conditions fulfilled there remains the possibility that the permeating substance may be actively transported at an equal rate in the two directions across the membrane. Because of the biological futility of membranes' engaging in such symmetrical active transport, this possibility can probably be disregarded.

TABLE III

*Simultaneous permeability coefficients in the two directions through the toad bladder*

| Hormone | No. of periods | Permeability coefficients ($\times 10^{-7}$ cm/sec) M→S* | S→M* | Δ | SE of Δ |
|---|---|---|---|---|---|
| Absent | 17 | 26.0 | 26.8 | 0.8 | ±2.3 |
| Present | 13 | 251.3 | 261.2 | 9.9 | ±7.9 |

* Mucosal to serosal and serosal to mucosal permeabilities measured simultaneously with $N^{15}$- and $C^{14}$-labeled urea.

there is no significant difference in the permeability coefficients in the two directions.

In an attempt to learn if carrier-mediated transport is involved in the enhanced flux of urea following hormone, studies were performed to determine if an increase in the concentration of unlabeled urea in the medium would result in depression of permeability to the labeled urea. Both labeled and unlabeled urea were added only to the mucosal medium. As can be seen in the last two columns of Table IV, increasing the concentration of urea to 25 or 50 mmoles was associated with a small decrease in the permeability coefficient as compared with values obtained during the control period, when only tracer amounts of urea were present in the medium. However, as a similar slight decrease with time is shown in the second and third columns, when no unlabeled urea was added, this decrease cannot be attributed to the added urea. The values for each period with or without added urea do not differ significantly.

*Site of hormonal action.* Evidence has been presented previously (15, 16) that the transmembrane permeability barrier presented by the toad bladder can be localized to two functionally distinct barriers located at or near the mucosal and serosal surfaces, respectively. The mucosal surface was found to be substantially less permeable to lactate ions than was the serosal surface. If these same barriers apply also to urea, the increased transmembrane permeability to urea occurring after hormone must be occasioned by an increase in the permeability of one or both diffusion barriers. Which of these is acted upon can be evaluated by comparing the concentration of $C^{14}$-urea in tissue water at the conclusion of flux experiments, performed with and without hormone. If labeled urea is placed in the medium bathing one side of the membrane (mucosal side in these experiments) the concentration of the labeled molecules in tissue water when a steady state is approximated will depend upon the relative permeability of the two surfaces. If permeability of the serosal surface is increased by hormone, what $C^{14}$-urea does pass through the mucosal barrier into the tissue will tend to pass out relatively more quickly across the serosal surface of the bladder treated with hormone. The concentration of $C^{14}$-urea in tissue water, therefore, will be lower in the hormone-treated than in the paired control bladder. If, on the other hand, the mucosal surface were made more permeable by the hormone, the labeled molecules would enter more quickly into the tissue water, hence the tissue water concentration of $C^{14}$-urea would be higher in the bladder treated with hormone than in the control. This is schematically depicted in Figure 2.

TABLE IV

*Absence of self-depression of high permeability to urea after hormone*

| Period | Without added urea $K_{trans}$* ($\times 10^{-7}$ cm/sec) | Without added urea Per cent of Period 1 | With added urea $K_{trans}$* ($\times 10^{-7}$ cm/sec) | With added urea Per cent of Period 1 |
|---|---|---|---|---|
| 1 | 334 | 100 | 336 | 100 |
| 2 | 292 | 88 | 321 | 95 |
| 3 | 270 | 81 | 262 | 78 |

* Mean of 6 experimental values per period. Nonlabeled urea added to mucosal medium at end of first period to yield concentrations of 25 (3 experiments) or 50 mmoles (3 experiments).

The technique of measurement using paired bladder halves has been described. The results are summarized in Table V. The per cent labeling of tissue water is listed for paired bladder halves without and with hormone added to the serosal medium, together with the average transmembrane permeability coefficient during the experiment. It will be seen that without hormone, labeling averaged 11 per cent whereas, coincident with the increased flux with the hormone, labeling rose in all experiments, to a mean of 38 per cent.

This increase in concentration of $C^{14}$-urea in tissue water was not a result simply of the attainment of a higher concentration of $C^{14}$-urea on the serosal side of the bladder treated with hormone. When the possible contribution from the serosal medium to the tissue activity is estimated, this

contribution amounts to one-twentieth or less of the observed total tissue activity and was subtracted from the tissue labeling before being included in Table V. In addition, for a direct check, two experiments (Experiments 4 and 5) were performed with sufficient $C^{14}$-urea added to the serosal bathing medium of the halves without hormone to bring the $C^{14}$-urea concentration up to or over that attained by the paired treated half, and the results were not significantly affected.

That the activity in the tissue in experiments with urea labeling was still present as urea, and not as some metabolic product, was demonstrated by treating several labeled tissue extracts with

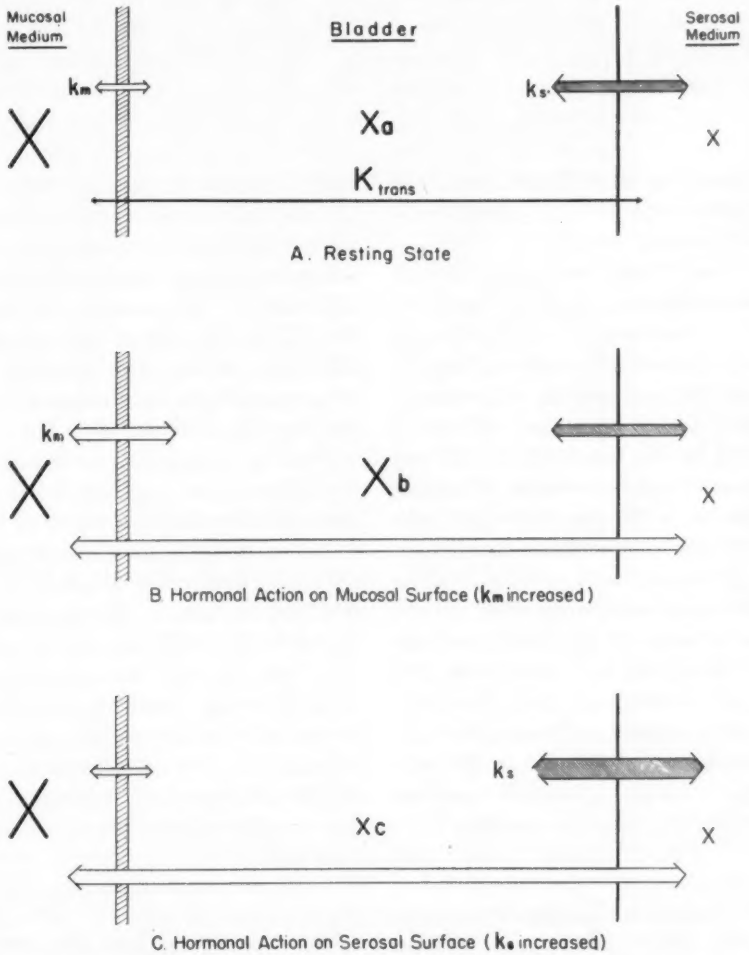



FIG. 2. SCHEMATIC DIAGRAM TO ILLUSTRATE METHOD OF DETERMINING AT WHICH SURFACE OF MEMBRANE THE NEUROHYPOPHYSEAL HORMONES ACT. Labeled urea, in these experiments, was added to the mucosal bathing medium to yield a concentration of tracer, X, in the medium. "A" represents the resting, non-hormone-treated state. "B" shows the increase in the transmembrane permeability coefficient, $K_{trans}$, and of the tissue concentration, $X_b$, that would result from an action of the hormone to increase the permeability of the mucosal diffusion barrier, $k_m$. A similar increase in $K_{trans}$ might be achieved by an action of the hormone to increase the serosal permeability, $k_s$, as is shown in "C", but such an effect would result in a reduction in tissue concentration, $X_c$, of the labeled urea. Hence the criterion for a mucosal site of action of the hormone is that $X_b > X_a$, and for a serosal site of action that $X_a > X_c$.

TABLE V

*Labeling of tissue water by $C^{14}$-urea from mucosal surface*

| Exp. | Min elapsed after $C^{14}$-urea added | Per cent labeling Hormone | | $K_{trans}$ ($\times 10^{-7}$ cm/sec) Hormone | |
|---|---|---|---|---|---|
| | | Absent | Present | Absent | Present |
| 1 | 72 | 22 | 44 | 22 | 418 |
| 2 | 75 | 7 | 44 | 15 | 265 |
| 3 | 78 | 6 | 47 | 14 | 227 |
| 4 | 89 | 5 | 35 | 5 | 300 |
| 5 | 90 | 16 | 34 | 30 | 503 |
| 6 | 112 | 5 | 43 | 7 | 255 |
| 7 | 122 | 14 | 23 | 11 | 324 |
| 8 | 156 | 11 | 37 | 36 | 340 |
| Mean | | 11 | 38 | 18 | 329 |

urease. By this means, at least 97 per cent of the activity of the tissue extracts disappeared, indicating it was in fact present as urea.

*Urea space.* When $C^{14}$-urea was applied to both surfaces of the bladder by immersing the tissue directly in a medium containing $C^{14}$-urea, the per cent labeling of tissue water obtained was significantly greater than 100 per cent in 42 measurements ($p = < 0.001$). It averaged $115 \pm 0.8$ (SE) per cent and varied from 103 to 125 per cent. This value could not be reduced by adding nonradioactive urea up to 300 mmoles to the bathing medium or by inhibiting metabolism through exposure to cold iodoacetic acid or to potassium cyanide. It was found to occur even when the tissue had been treated with 10 per cent trichloroacetic acid or by boiling prior to incubation in $C^{14}$-urea. After treatment with urease the tissue radioactivity disappeared completely. Presence or absence of hormone made no difference in the percentage of labeling. Similar high values were obtained with $C^{14}$-thiourea, whereas another $C^{14}$-labeled compound, lactate, labeled nearly 100 per cent of tissue water, as did tritiated water. With other toad tissues the urea space was lower: heart and colon, 100 per cent; muscle, 105 per cent; but skin reached 109 per cent. We are unable to explain these findings of high labeling at this time although it is of interest that high values have also been reported for mammalian red blood cells (17) and for frog muscles (18).

TABLE VI

*Osmolalities and urea concentrations of toad serum and urine*

| | Urea concentration (mmoles/L) | | Total solute concentration (mOsm/kg $H_2O$) | |
|---|---|---|---|---|
| | Serum | Urine | Serum | Urine |
| Individual toads | 44 | 43 | | 302 |
| | 39 | 40 | 297 | 287 |
| | 24 | 38 | 224 | 126 |
| | 21 | 39 | 204 | 148 |
| Pooled measurements | | | | |
| 6 toads | 18 | 46 | 230 | 105 |
| 5 toads | 15 | 42 | 218 | 86 |

*Urine urea and state of hydration.* To permit evaluation of the significance to the intact toad of these *in vitro* findings for urea permeability, the results of simultaneous measurements of concentrations of urea and total solutes in blood and urine of toads in random states of water balance are shown in Table VI. It can be seen that the urine urea concentration remains relatively constant, whereas, using osmolality of serum and urine as indices of hydration, serum urea drops with hydration and rises with dehydration, when it approaches identity with urine. The implications of these findings to the water economy of the toad will be discussed.

## DISCUSSION

These studies indicate that urea penetrates the toad bladder by passive diffusion, and that this diffusion is markedly increased by neurohypophyseal hormones, which act to increase the permeability of a barrier at or near the mucosal surface.

There are a few animal species in which active transport of urea by the renal tubule appears to take place. Active urea secretion has been shown in the bullfrog (6–8) and has been suggested in the case of certain rodents (19), while active reabsorption of urea occurs in elasmobranchs (20). In general, however, the movement of urea across

the mammalian renal tubular epithelium has been considered to be by free diffusion (21–23) as our studies indicate to be the case for the toad bladder.

The equal permeability to urea in both directions across the membrane indicates its passage by a passive process. Even in the absence of active transport the possibility of a carrier-mediated transport was considered. When, however, the concentration of unlabeled urea was increased to 50 mmoles in the mucosal bathing medium (a concentration comparable with that present in the bladder urine of the toad *in vivo*) no evidence of self-depression of the permeability to $C^{14}$-urea was detected. However, at these concentrations of urea a "carrier" might still be incompletely saturated. Urea is known to form hydrogen bonds with great facility (24). For example, one molecule of mercaptoalbumin has been estimated to bind some 2,000 urea molecules (25) or double its own weight. Hence, if a carrier for urea actually existed, which depended upon hydrogen bonding, its capacity might conceivably be quite large, and the concentrations of urea utilized in our study might then have been too low to test adequately for competition. To exclude this possibility, studies at much higher urea concentrations would have to be performed. When higher ambient urea concentrations were employed, however, interpretation of the results was complicated by apparent deterioration of the membrane.

It appears most likely that urea moves through the toad bladder in aqueous channels. This is suggested by the solubility properties of the compound; urea is much more soluble in water than in lipid solvents. Collander and Bärlund (26) found the partition coefficients for urea in ether: water and olive oil: water systems to be 0.00047 and 0.00015, respectively. Similar values (averaging 0.0007) have been obtained in this laboratory with $C^{14}$-urea in a hexane: Ringer solution system. Also consistent with the notion that urea moves in aqueous channels are the observations regarding the relative permeability coefficients for water and urea through the membrane. In the absence of hormone, the permeability coefficient for urea is low, approximating that of a large group of substances tested. Water penetrates at a much greater rate, approximately $900 \times 10^{-7}$ cm per second. With hormone, however, the percentage increase in permeability to urea rises sharply, much more than does that to water. Hence, the ratio of the permeability coefficients for urea and water, which was approximately 1 : 36 before hormone, rises to 1 : 6 after hormone. At 25° C the aqueous free diffusion coefficient for urea is $137 \times 10^{-7}$ cm² per second (27), and for water is $246 \times 10^{-7}$ cm² per second [3] (28) or a ratio of 1 : 2. It is thus apparent that in the presence of hormone the ratio of the permeabilities to urea and water begins to approach that expected if both are penetrating the membrane by diffusion through aqueous channels.

Koefoed-Johnsen and Ussing (11) have presented convincing evidence that neurohypophyseal hormones increase the permeability of the toad skin to water by dilating aqueous pores. If such is also the case for the toad bladder and if it is assumed that, following hormone, channels previously accessible only to water become accessible also to urea so that the bulk of the post-hormonal diffusion is occurring through these dilated channels, the data indicate that urea is hindered in these channels to a greater extent than is water, by a factor of about three. If, on the other hand, one makes the assumption that the effect of the hormone is just to open up or otherwise make available channels previously inaccessible to both urea and water, then the increase in permeability occurring with the hormone will reflect the relative ease with which the two molecules diffuse through these new channels. The increases found were for urea, $248 \times 10^{-7}$ cm per second and for water, $700 \times 10^{-7}$ cm per second or a ratio of 1 : 2.8, which approaches the 1 : 2 ratio of their free diffusion coefficients.

The increase in the transmembrane permeability and in tissue labeling with $C^{14}$-urea which occurs across the mucosal surface following the administration of hormone indicates that the relative permeability of a mucosal barrier has been increased. We may ask whether this is an effect purely on a mucosal barrier, or whether in addition there has

[3] The fact that the permeability coefficient and the free diffusion coefficient are in the same absolute range does not mean that urea and water are unhindered by the membrane. Diffusion coefficients are based on a barrier thickness of 1 cm of solvent, whereas permeability coefficients ignore the thickness of the barrier. Since the mucosa of the distended toad bladder may be approximately 30 $\mu$ (0.003 cm) thick, its "diffusion resistance" to urea in the presence of hormone is still some 175 times greater than that of a comparable thickness of water.

been a change in the permeability of the serosal barrier. This question can be approached, but not resolved at the present time, by a more detailed consideration of the two permeability barriers and their relation to the transmembrane permeability.

If the mucosal bathing medium is labeled and the labeled substance penetrates the membrane passively, then when a steady state is reached the net fluxes of the substance across the mucosal permeability barrier, across the serosal permeability barrier, and across the entire membrane, will be equal. That is,

$$(C_m - C_c)\, k_m = (C_c - C_s)\, k_s = (C_m - C_s)\, K_{trans},$$

where $C_m$, $C_s$, and $C_c$ are the concentrations of the substance in the mucosal medium, the serosal medium, and the cell water contained within the barriers, respectively, and $k_m$, $k_s$, and $K_{trans}$ are the permeability coefficients of the mucosal barrier, the serosal barrier, and the entire membrane, respectively. Since $C_s$ is essentially zero, the second and third equalities reduce to

$$C_c k_s = C_m K_{trans}. \quad \text{Rearranging, } k_s = \frac{K_{trans}}{\frac{C_c}{C_m}}.$$

We have termed the fraction $\frac{C_c}{C_m} \times 100$ the per cent labeling from the mucosal surface.

$$\text{Therefore, } k_s = \frac{K_{trans}}{\text{per cent labeling}} \times 100.$$

A similar expression for $k_m$ can be derived:

$$k_m = \frac{K_{trans}}{100 \text{ per cent labeling}} \times 100.$$

The presence or absence of change in $k_s$ following hormone will therefore be reflected in the relative change of $K_{trans}$ to that of the per cent labeling. However, arriving at an accurate value for $k_s$ requires in turn an accurate value for $C_c$, the concentration of $C^{14}$-urea in the tissue water within the two permeability barriers, and several complicating factors obscure the latter's true value:

1. There is a significant inulin space on the mucosal aspect of the bladder which is in equilibrium with the labeled urea of the mucosal medium. Thus, mucosal labeling studies with $C^{14}$-inulin performed in a manner identical with those for $C^{14}$-urea have revealed a variable labeling (unaffected by hormone) of 0 to 16 per cent, averaging 7 per cent in nine experiments. Presumably this is all external to the mucosal permeability barrier, since inulin characteristically occupies an extracellular position in tissues. Hence the per cent labeling of urea which is applicable in the equation for $k_s$ is too large by this variable amount. If the urea labeling is reduced by this amount the "corrected" urea labeling without hormone is only a small percentage and the value for $k_s$ is markedly increased. This correction will have a much smaller affect on $k_s$ when urea labeling is high in the bladder treated with hormone.

2. The inulin space on the serosal surface averages approximately 40 per cent of tissue water. This amount of tissue water is also very likely outside the permeability barrier, and its concentration of $C^{14}$-urea probably approaches that of the serosal bathing medium, and hence may be close to zero. Consequently the concentration of urea between the permeability barriers may actually be up to 40 per cent greater than is calculated.

3. The labeling of whole bladder water of greater than 100 per cent adds uncertainty to the concentration of free $C^{14}$-urea in the intracellular water in the mucosal labeling experiments.

Because of these factors absolute values for the individual permeability coefficients $k_m$ and $k_s$ before and after hormone, can not be calculated with confidence. However, taking into account the complicating factors, an estimate of the relative change occurring in $k_m$ and $k_s$ upon the administration of the hormone can be made. Subtracting the average mucosal inulin space (7 per cent) from the average per cent labeling with urea before (11 per cent) and after (38 per cent) hormone, corrected values of 4 per cent before and 31 per cent after are obtained. Substituting these figures into the appropriate equations gives $k_m$, $19 \times 10^{-7}$ cm per second before, and $477 \times 10^{-7}$ cm per second after, or an increase in the range of 25-fold; and $k_s$, $450 \times 10^{-7}$ cm per second before, and $1{,}061 \times 10^{-7}$ cm per second after, or an increase of possibly twofold. Correcting for the serosal inulin space would not change the relative increase of $k_s$ and would increase still further that of $k_m$.

Thus the action of hormone on the permeability barriers of the toad bladder to urea can be characterized as follows: when the hormone is absent, the low transmembrane penetration of urea is due

primarily to the relative impermeability of a mucosal barrier. When hormone is added to the serosal surface, the permeability of this mucosal barrier increases strikingly and a large increase in transmembrane permeability to urea results. Whether in addition, a much smaller relative increase in permeability of the serosal surface contributes to the large post-hormonal transmembrane permeability cannot be ruled in or out on the basis of the present studies.

It seems entirely possible that the mucosal action of the hormone may prove to be its sole action not only with respect to urea but also in the case of other substances whose permeability it affects, notably water and sodium. Preliminary studies employing tritium-labeled water show a significant increase in tissue water labeling across the mucosal barrier following hormone, again indicating an important change in mucosal permeability. A similar result has been obtained with sodium; here, however interpretation is more difficult, since sodium is a charged particle, and its concentration in tissue water following hormone may be affected by changes in electrical potentials across the cell membranes as well as by changes in membrane permeability.

The mucosal site of action of the hormone is all the more striking when one considers that the hormone acts only when it is added to the serosal bathing medium. This may simply be another consequence of the impermeability of the mucosal surface which prevents access of the hormone to its site of action from that side.

At the present time the action of the hormone appears to affect the permeability to only a few substances: sodium, water, urea and certain analogs of the latter. The lack of an effect on thiourea emphasizes the specificity of the permeability changes induced in the membrane by the hormone. An important property common to water, urea and acetamide, but not to thiourea, is the marked propensity of the former group to undergo hydrogen bonding. Recently, Stein and Danielli (29) and Stein (30) have presented evidence that hydrogen bonding accounts for the facilitated diffusion of glycerol through the membranes of red blood cells. They postulate that, in the red cell, bimolecular protein layers penetrate through the double lipid layer comprising the cell membrane. These bimolecular protein layers could thus serve as channels accessible to compounds whose ability to hydrogen bond allows them to slip between the two layers of hydrogen-bonded protein molecules. According to this view the action of the hormone might be to open such protein-lined channels at the mucosal surface making them available for entry by suitable molecules. As mentioned, the present study failed to test critically for any mechanism of penetration which is dependent upon hydrogen bonding. Furthermore, how this view may be reconciled with evidence for bulk flow of water through this membrane (31) and how the sodium ion can have access to such channels, are problems which remain unresolved by this view of the membrane.

Andersen and Ussing (12) observed a marked augmentation of the permeability of the isolated skin of the toad, *Bufo bufo,* to thiourea after treatment with neurohypophyseal hormones. This finding is in apparent contradiction to our failure to observe any change in permeability of the toad bladder to thiourea following hormone. However, when the effect of hormone on the isolated skin of the toad, *Bufo marinus,* used in the present study, was tested with respect to urea, thiourea and sodium, no effect on either urea or thiourea permeability was observed, although the usual large stimulation of sodium transport occurred. This emphasizes the differences which may exist in analogous membranes from closely related species.

The increased permeability of the toad bladder to urea in the presence of neurohypophyseal hormones may have importance for the water economy of the animal. Ewer (32) first noted that the large volume of fluid contained within the urinary bladder of the toad served as a reserve supply of water which was rapidly reabsorbed during dehydration or following administration of mammalian posterior pituitary extracts. Such fluid reabsorption in response to hormone requires the presence of an osmotic gradient across the membrane, as has been shown for the frog and toad skins (11, 33) and recently by Bentley (34) for the toad bladder *in vitro.* Active sodium reabsorption will reduce urine osmolality and afford the requisite osmotic gradient. In the well hydrated toad, final urine sodium concentrations of only a few milliequivalents per liter have been found with dilute urines of 50 to 100 mOsm per kg water (13). Of the solute now contributing to the

urine osmolality, 50 per cent may be urea, and its concentration in the urine considerably exceeds that in the serum. If an action of neurohypophyseal hormone, in addition to facilitating water reabsorption from such a urine, were to increase the permeability of the bladder to urea as well, then back diffusion of urea through the bladder wall would serve to sustain the osmotic gradient for reabsorption of water. This would result in a considerable economy of water to the toad at the expense of an elevation of blood urea. Table VI shows that the dehydrated toad excretes a urine essentially isosmotic with its serum and that the urine urea and serum urea concentrations become equal. Hence diffusion equilibrium is attained between urine and serum for both water and urea.

The saving in water to the animal by this reabsorption of urea can be estimated. If urea were impenetrant, approximately 75 per cent of the water in the bladder could be reabsorbed before the osmotic effect of retained urea and other urinary solutes would prevent further reabsorption of water. With urea also moving out of the bladder, this figure would rise to 95 per cent, or a 20 per cent gain of water reabsorbed. Regarded in terms of the water remaining unavailable in the bladder, this represents a reduction of 80 per cent in the water lost to the toad by urination.

Similar considerations may apply to the more complex mammalian renal tubule. Klümper, Ullrich and Hilger (23) and Levinsky and Berliner (4) have recently offered evidence that, during the elaboration of concentrated urine, both water and urea leave the collecting tubule. The concentration of urea in the medullary interstitial fluid increases thereby and balances osmotically its concentration in the collecting tubule. Thus, a high permeability to urea postulated for the collecting tubule epithelium allows urea to equilibrate on the two sides of the epithelium and the high nonurea solute concentration of the medullary interstitial fluid can exert its full osmotic force to concentrate the nonurea solute of the urine.

The high permeability of the collecting tubule to urea, necessary for such equilibration to occur during antidiuresis, may result from a direct action of antidiuretic hormone. The increased back reabsorption of urea which occurs during antidiuresis has been attributed in the past to the lower rate of urine flow, and the possibility of an additional effect of antidiuretic hormone on the permeability of the mammalian renal tubule to urea has not as yet been definitely shown, but is suggested by observations of Shannon (35). He found that the ratio of the urea to creatinine clearances may be depressed as low as 0.20 in dogs deprived of water for at least 24 hours, whereas this ratio was 0.55 to 0.65 at similar rates of urine flow from dogs that were less dehydrated and in which presumably lower levels of circulating antidiuretic hormone existed. It remains to be determined whether neurohypophyseal hormones directly affect the permeability of the mammalian renal tubule to urea as has been demonstrated in this study for the isolated toad bladder.

## SUMMARY

1. The effect of mammalian neurohypophyseal hormones on the permeability of the isolated toad bladder to urea has been determined.

2. The hormone added to the serosal bathing medium increases the permeability coefficient from $26 \pm 2.5$ (SE) $\times 10^{-7}$ cm per second to $274 \pm 3.8$ (SE) $\times 10^{-7}$ cm per second.

3. The permeabilities measured simultaneously in the two directions across the membrane with $C^{14}$- and $N^{15}$-urea, respectively, were found equal both in the presence and absence of hormone. This indicates that urea moves passively through the bladder.

4. No evidence of self-depression of the permeability to urea was detected with concentrations of urea in the medium up to 50 mmoles. Although this suggests that there is no carrier-mediated transport through the tissue, it would not exclude the possibility of such transport if urea were hydrogen-bonded to the "carrier."

5. Although the hormonal action on urea permeability affects a passive process, its action, nevertheless, is highly specific. This is illustrated by the absence of an effect of the hormone on the permeability of the bladder to thiourea.

6. The increase in permeability of the membrane to urea following hormone can be largely, if not entirely, accounted for by an action of the hormone to increase the permeability of a diffusion barrier at the mucosal surface of the membrane.

## REFERENCES

1. Hargitay, B., and Kuhn, W. Das Multiplikationsprinzip als Grundlage der Harnkonzentrierung in der Niere. Z. Elektrochem. 1951, **55**, 539.

2. Miles, B. E., Paton, A., and de Wardener, H. E. Maximum urine concentration. Brit. med. J. 1954, **2**, 901.
3. Epstein, F. K., Gleeman, C. R., Pursel, S., and Hendrikx, A. The effect of feeding protein and urea on the renal concentrating process. J. clin. Invest. 1957, **36**, 635.
4. Levinsky, N. G., and Berliner, R. W. The role of urea in the urine concentrating mechanism. J. clin. Invest. 1959, **38**, 741.
5. Crawford, J. D., Doyle, A. P., and Probst, J. H. Service of urea in renal water conservation. Amer. J. Physiol. 1959, **196**, 545.
6. Marshall, E. K., Jr., and Crane, M. M. The secretory function of the renal tubules. Amer. J. Physiol. 1924, **70**, 465.
7. Forster, R. P. Active cellular transport of urea by frog renal tubules. Amer. J. Physiol. 1954, **179**, 372.
8. Love, J. K., and Lifson, N. Transtubular movements of urea in the perfused bullfrog kidney. Fed. Proc. 1958, **17**, 100.
9. Ussing, H. H., and Zerahn, K. Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. Acta physiol. scand. 1951, **23**, 110.
10. Ussing, H. H. The distinction by means of tracers between active transport and diffusion. The transfer of iodide across the isolated frog skin. Acta physiol. scand. 1949, **19**, 43.
11. Koefoed-Johnsen, V., and Ussing, H. H. The contributions of diffusion and flow to the passage of $D_2O$ through living membranes. Effect of neurohypophyseal hormone on isolated anuran skin. Acta physiol. scand. 1953, **28**, 60.
12. Andersen, B., and Ussing, H. H. Solvent drag on non-electrolytes during osmotic flow through isolated toad skin and its response to antidiuretic hormone. Acta physiol. scand. 1957, **39**, 228.
13. Leaf, A., Anderson, J., and Page, L. B. Active sodium transport by the isolated toad bladder. J. gen. Physiol. 1958, **41**, 657.
14. Sprinson, D. B., and Rittenberg, D. The rate of utilization of ammonia for protein synthesis. J. biol. Chem. 1949, **180**, 707.
15. Leaf, A. Measurement of the permeability of the two surfaces of a living membrane. Science 1958, **128**, 144.
16. Leaf, A. The mechanism of the asymmetrical distribution of endogenous lactate about the isolated toad bladder. J. cell. comp. Physiol. 1959, **54**, 103.
17. Parpart, A. K., and Shull, J. C. Solvent water in the normal mammalian erythrocyte. J. cell. comp. Physiol. 1935, **6**, 137.
18. Bozler, E. Osmotic effects and diffusion of non-electrolytes in muscle. Amer. J. Physiol. 1959, **197**, 505.
19. Schmidt-Nielsen, B. Urea excretion in mammals. Physiol. Rev. 1958, **38**, 139.
20. Smith, H. W. Absorption and excretion of water and salts by the elasmobranch fishes. II. Amer. J. Physiol. 1931, **98**, 296.
21. Rehberg, P. B. Studies on kidney function. II. The excretion of urea and chlorine analysed according to a modified filtration-reabsorption theory. Biochem. J. 1926, **20**, 461.
22. Chasis, H., and Smith, H. W. The excretion of urea in normal man and in subjects with glomerulonephritis. J. clin. Invest. 1938, **17**, 347.
23. Klümper, J. D., Ullrich, K. J., and Hilger, H. H. Das Verhalten des Harnstoffs in den Sammelrohren der Säugetierniere. Pflüg. Arch. ges. Physiol. 1958, **267**, 238.
24. Pauling, L. The Nature of the Chemical Bond, 2nd ed. London, Oxford University Press, 1948, p. 315.
25. Kay, C. M., and Edsall, J. T. Dimerization of mercaptalbumin in presence of mercurials. III. Bovine mercaptalbumin in water and in concentrated urea solutions. Arch. Biochem. 1956, **65**, 354.
26. Collander, R., and Bärlund, H. Permeabilitäts Studien an *chara ceratophyllia*. Acta botanica fenn. 1933, **11**, 1.
27. Gosting, L. J., and Akeley, D. F. A study of the diffusion of urea in water at 25° with the Gouy interference method. J. Amer. chem. Soc. 1952, **74**, 2058.
28. Wang, J. H., Robinson, C. V., and Edelman, I. S. Self-diffusion and structure of liquid water. III. Measurement of the self-diffusion of liquid water with $H^2$, $H^3$, and $O^{18}$ as tracers. J. Amer. chem. Soc. 1953, **75**, 466.
29. Stein, W. D., and Danielli, J. F. Structure and function in red cell permeability *in* Membrane Phenomena, A General Discussion of the Faraday Society. Aberdeen, The University Press, 1956, p. 250.
30. Stein, W. D. N-terminal histidine at the active centre of a permeability mechanism. Nature (Lond.) 1958, **181**, 1662.
31. Hays, R. Unpublished data.
32. Ewer, R. F. The effect of pituitrin on fluid distribution in *Bufo regularis* Reuss. J. exp. Biol. 1952, **29**, 173.
33. Sawyer, W. H. Effect of posterior pituitary extract on permeability of frog skin to water. Amer. J. Physiol. 1951, **164**, 44.
34. Bentley, P. J. The effects of neurohypophyseal extracts on water transfer across the wall of the isolated urinary bladder of the toad *Bufo marinus*. J. Endocr. 1958, **17**, 201.
35. Shannon, J. A. Glomerular filtration and urea excretion in relation to urine flow in the dog. Amer. J. Physiol. 1936, **117**, 206.

# STUDIES ON THE SITE OF THE FEEDBACK CONTROL OF CHOLESTEROL SYNTHESIS * †

By MARVIN D. SIPERSTEIN and M. JOANNE GUEST

*(From the Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas)*

(Submitted for publication August 21, 1959; accepted December 4, 1959)

Despite wide variations in dietary cholesterol, most animals are capable of maintaining the cholesterol of their plasma at relatively constant levels. Insight into the means by which this homeostatis of plasma cholesterol is accomplished was first provided when Gould and associates (3–5), Tomkins, Sheppard and Chaikoff (6) and Frantz, Schneider and Hinkelman (7) independently demonstrated that cholesterol synthesis in the liver varies inversely with the level of dietary cholesterol. Since all endogenous plasma cholesterol probably originates in the liver (8–10), it is likely that the constancy of plasma cholesterol is the result of this reciprocal relationship between dietary cholesterol and hepatic cholesterogenesis.

The mechanism by which exogenous cholesterol is able to suppress cholesterol synthesis in the liver is not as yet understood. Previous studies of this problem have been carried out by Gould and Popják (11) who presented preliminary evidence indicating that the conversion of mevalonic acid to cholesterol in liver homogenates is not impaired by cholesterol feeding. Similarly, since completion of our study, Bucher, McGarrahan, Gould and Loud (12, 13), employing an approach similar to that used here, have clearly demonstrated that in homogenates the reactions involved in the conversion of mevalonic acid to cholesterol are not greatly influenced by dietary cholesterol. These experiments (11–13), as well as the present investigation, provide evidence that the site of control of cholesterol synthesis is located proximal to the utilization of mevalonic acid.

The present report describes studies which specifically localize the biochemical site of the cholesterol-induced inhibition of cholesterol synthesis. Some of the properties of this negative feedback system are described, and it is suggested, on the basis of these characteristics, that this type of feedback control may be a more widespread means of regulating synthetic processes than has previously been realized.

## EXPERIMENTAL PROCEDURE

Male and female rats of the Long-Evans and Sprague-Dawley strains, weighing between 150 and 250 g, were used in this study. They were maintained on a commercial chow prior to initiation of the experiment. During the experimental period, the control animals were fed *ad libitum* either a powdered commercial animal chow or Simonson "White Diet" (Simonson Laboratories, Gilroy, Calif.); the experimental rats received the same diets, to which cholesterol was added in 0.5, 2.5 or 5 per cent amounts. Cholesterol in these concentrations did not influence the food intake of the rats.

The rats were killed by a blow on the head and exsanguinated. The livers, and in some cases the intestines, were excised and cooled. One mm slices of these organs were prepared by means of a McIlwain tissue slicer, and duplicate 500 mg portions of the slices were placed in the outer wells of 25 ml center-well flasks containing 5 ml of Krebs-Henseleit bicarbonate buffer, pH 7.4, and the $C^{14}$-labeled substrate. The flasks were then gassed with a 5 per cent carbon dioxide, 95 per cent oxygen mixture and incubated for 3 hours at 37° C in a Dubnoff metabolic shaker.

Acetate-1-$C^{14}$ and acetate-2-$C^{14}$ were purchased from Nuclear-Chicago Corp.; mevalonic acid-2-$C^{14}$ was obtained from Isotopic Specialties Co.; and labeled squalene was biologically synthesized by incubating liver slices from normal rats with mevalonic acid-2-$C^{14}$ and isolating the synthesized squalene-$C^{14}$ as described below.

## ANALYTICAL METHODS

*Isolation of $C^{14}O_2$ and nonsaponifiable material.* Carbon dioxide was trapped in the center well of the flask with Hyamine 10 X as previously described (14). Following removal of the $CO_2$, the contents of the main compartment were saponified by adding 0.5 ml of 90 per

* The results reported in this paper were presented at the meeting of the Southern Society for Clinical Investigation held in New Orleans on January 19, 1959 and have appeared in abstract form (1, 2).

† This research was supported by grants from the Dallas Heart Association, the American Heart Association and the Texas Heart Association.

cent potassium hydroxide and autoclaving for 1 hour. The nonsaponifiable material was next isolated by transferring the contents of the center-well flask to a 250 ml Erlenmeyer flask, adding an equal volume of absolute ethyl alcohol and extracting twice with at least a tenfold excess of petroleum ether.

*Isolation of squalene.* The petroleum ether solution containing both squalene and cholesterol was taken to dryness under nitrogen to remove ethyl alcohol and the residue was dissolved in pentane; 100 mg of squalene (Eastman Organic Chemicals), redistilled at 3 mm Hg as described by Heilbron, Kamm and Owens (15), was added to an aliquot of the pentane solution, and the mixture was then transferred to a silicic acid column (10 g of unactivated Baker reagent grade silicic acid in a $2.5 \times 23$ cm column). The squalene was eluted with 100 ml of petroleum ether. An aliquot of this solution was added directly to 5 ml of 0.4 per cent diphenyloxazole in toluene and the $C^{14}$ content of the squalene determined in a Packard liquid scintillation counter.

The validity of this method of isolation of squalene-$C^{14}$ was established by forming the hexachloride derivative of the isolated squalene-$C^{14}$ using the method of Heilbron and colleagues (15). The squalene hexachloride was washed at $-20°$ C 3 times each with ethanol and with ethyl ether. The resulting white powder was dried, dissolved directly in 5 ml of 0.4 per cent diphenyloxazole in toluene and assayed for $C^{14}$ in the liquid scintillation counter. It was found that if the above procedure was followed, at least 95 per cent of the $C^{14}$ obtained in the petroleum ether eluate of the silicic acid was recoverable as the squalene hexachloride. On the other hand, if at any stage the extracted squalene was exposed to air in the dry state for a day or more, significant losses occurred.

*Cholesterol-$C^{14}$ determination.* The fraction containing the cholesterol-$C^{14}$ was eluted from the silicic acid column with ethyl ether, and the cholesterol was then isolated as its digitonide by the procedure of Sperry and Webb (16). The cholesterol digitonide was dissolved in 2 ml of methanol and its $C^{14}$ content assayed as described above.

*Fatty acid-$C^{14}$ and ketone body-$C^{14}$ determination.* The aqueous residue obtained after removal of the nonsaponifiable material was heated on a steam bath to remove the ethanol following which the pH of the solution was brought to 3.0 with sulfuric acid. Exactly 40 ml of pentane was next added and the flask was shaken for 15 minutes on an International bottle shaker to extract the free fatty acids. The pentane layer was then removed, washed twice with water and a 4 ml aliquot added to 12 ml of the diphenyloxazole-toluene for $C^{14}$ determination.

To assay the acetoacetic acid-$C^{14}$ and $\beta$-hydroxybutyric acid-$C^{14}$, carrier ketone bodies were added to a deproteinized extract of the flask contents, and the method described by Van Slyke (17) was employed to isolate the corresponding Denigès's salt. These were washed twice with 5 ml portions of cold water, mounted on filter paper and their $C^{14}$ content assayed at infinite thickness in a Geiger counter equipped with a micromil window.

The following data represent, in every case, the average value obtained from the analysis of duplicate experiments.

## RESULTS

### *Influence of cholesterol feeding on cholesterol synthesis from acetate*

In confirmation of previous studies (3–7), the feeding of a 2.5 or a 5 per cent cholesterol diet resulted in a striking depression in the synthesis of cholesterol from acetate by rat liver slices. The magnitude of decrease in cholesterol synthesis, however, was quite variable. In the typical experiments shown in Table I, cholesterol synthesis was reduced by a factor of from 21 to 150. In other determinations presented in Tables IV and VI, cholesterol feeding inhibited cholesterogenesis by a factor of from 10 to 300.

Frantz and colleagues (7) have reported that this depression in cholesterogenesis can be correlated with the extent of accumulation of cholesterol

TABLE I

*Effect of cholesterol feeding on cholesterol synthesis*

| Experiment | Cholesterol in diet | | Liver cholesterol | Acetate-$C^{14}$ converted to cholesterol |
|---|---|---|---|---|
| | % | *Duration* | *mg/100 g* | % |
| 1 | 0 | 3 days | 214 | 1.430 |
| | 2.5 | 3 days | 206 | 0.010 |
| 2 | 0 | 1 month | 176 | 0.272 |
| | 5 | 1 month | 154 | 0.013 |
| 3 | 0 | 3 days | 218 | 0.572 |
| | 2.5 | 3 days | 276 | 0.004 |
| 4 | 0 | 1 day* | 233 | 3.460 |
| | 10 | 1 day† | 250 | 0.043 |

* Tube-fed 5 ml of corn oil, 12 and 24 hours prior to killing.
† Tube-fed 0.5 g of cholesterol in 5 ml of corn oil, 12 and 24 hours prior to killing.

in the liver and, in fact, a logarithmic response to increases in cholesterol concentration was found by these authors. In our studies, on the other hand, dietary cholesterol produced a marked depression in cholesterol synthesis (reduced by a factor of at least 100 in Experiments 1 and 3, Table I) prior to the appearance of a detectable elevation in the concentration of hepatic cholesterol. The reason for this greater sensitivity to cholesterol feeding is not apparent; however, it is obvious that the mechanism by which cholesterol synthesis is depressed by dietary cholesterol need not be dependent upon large increases in tissue cholesterol concentration.

### *Preliminary localization of the site of the feedback control of cholesterogenesis*

According to present knowledge, the conversion of acetate to cholesterol involves about 26 separate enzymatic reactions. The approximate localization of the site at which exogenous cholesterol produces its inhibition of cholesterol synthesis was determined by separately examining the influence of cholesterol feeding on the various segments of this pathway, indicated in Figure 1.

*Influence of cholesterol feeding on the conversion of squalene to cholesterol.* Approximately 15 steps are thought to be involved in the conversion of squalene to cholesterol (18). As demonstrated by the experiments shown in Table II, cholesterol feeding has little or no demonstrable influence on this series of reactions. Wide variations in the rates of incorporation of the labeled squalene into cholesterol by rat liver slices were observed; however, in no case was the magnitude of decrease sufficient to account for the reduction, by a factor of 10 to 300, in the conversion of *acetate* to cholesterol which results from cholesterol feeding.

*Conversion of mevalonic acid to squalene.* Seven reactions are believed to be required for the conversion of mevalonic acid to squalene (19, 20). In order to determine whether dietary cholesterol causes an inhibition of one or more of these reactions, the conversion of mevalonic acid-2-$C^{14}$ to squalene was examined in the livers of both normal and cholesterol-fed rats.

As is shown in Table III, cholesterol feeding consistently increased, rather than depressed, the synthesis of squalene from mevalonic acid; the site of cholesterol inhibition is, therefore, not located in this segment of cholesterol synthesis.

*Conversion of mevalonic acid to cholesterol.* The results of the previous two experiments would indicate that the 22 reactions necessary for the synthesis of cholesterol from mevalonate are not inhibited by cholesterol feeding. This conclusion was further tested by studying the conversion of mevalonic acid-2-$C^{14}$ to cholesterol. In each experiment, the synthesis of cholesterol from acetate was also examined, using another sample of the pooled liver slices obtained from the

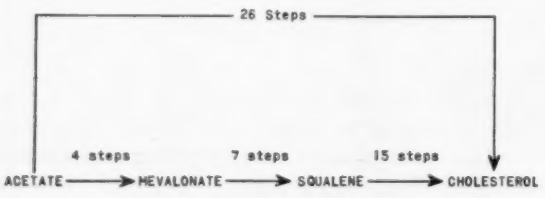



FIG. 1. SUMMARY OF REACTIONS OF CHOLESTEROL SYNTHESIS.

TABLE II

*Effect of cholesterol feeding on the synthesis of cholesterol from squalene* *

| Experiment | Cholesterol in diet | Squalene incorporated into cholesterol |
|---|---|---|
| | % | % |
| 1 | 0 | 0.75 |
| | 2.5 | 1.14 |
| 2 | 0 | 1.14 |
| | 2.5 | 0.35 |
| 3 | 0 | 0.94 |
| | 2.5 | 0.54 |
| 4 | 0 | 0.85 |
| | 2.5 | 0.71 |

* Conditions: Experiments 1 and 2, squalene-$C^{14}$ dissolved in 0.1 ml acetone. Experiments 3 and 4, squalene-$C^{14}$ suspended in 1 drop of Tween 80, and added to 500 mg liver slices in 5 ml Krebs-phosphate buffer.

TABLE III

*Effect of cholesterol feeding on the conversion of mevalonic acid to squalene*

| Experiment | Cholesterol in diet | | Mevalonic acid converted to squalene |
|---|---|---|---|
| | % | *Duration* | % *added* $C^{14}$ |
| 1 | 0 | 9 days | 3.02 |
| | 5 | 9 days | 8.11 |
| 2 | 0 | 8 days | 1.72 |
| | 2.5 | 8 days | 9.04 |
| 3 | 0 | 1 month | 0.17 |
| | 5 | 1 month | 0.65 |
| 4 | 0 | 10 days | 0.32 |
| | 5 | 10 days | 6.95 |

TABLE IV

*Effect of cholesterol feeding on cholesterol synthesis from acetate and mevalonic acid*

| Exp. | Cholesterol in diet | | Liver | | Intestine | |
|---|---|---|---|---|---|---|
| | | | Acetate-1-$C^{14}$ | Mevalonic acid-2-$C^{14}$ | Acetate-1-$C^{14}$ | Mevalonic acid-2-$C^{14}$ |
| | % | *Duration* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* |
| 1 | 0 | 3 days | 0.535 | 10.81 | | |
| | 0.5 | 3 days | 0.032 | 6.56 | | |
| | 5 | 3 days | 0.005 | 3.62 | | |
| 2 | 0 | 9 days | 0.970 | 1.91 | 0.278 | 0.045 |
| | 0.5 | 9 days | 0.635 | 1.58 | 0.353 | 0.068 |
| | 5 | 9 days | 0.011 | 0.66 | 0.117 | 0.049 |
| 3 | 0 | 3 days | 0.248 | 1.18 | 0.160 | 0.144 |
| | 0.5 | 3 days | 0.208 | 0.76 | 0.077 | 0.097 |
| | 5 | 3 days | 0.008 | 0.30 | 0.223 | 0.258 |
| 4 | 0 | 3 days | 0.624 | 8.64 | | |
| | 0.5 | 3 days | 0.081 | 8.27 | | |
| | 5 | 3 days | 0.008 | 2.69 | | |
| 5 | 0 | 3 days | 2.536 | 2.12 | 0.106 | 0.011 |
| | 0.5 | 3 days | 2.328 | 2.50 | 0.054 | 0.013 |
| | 5 | 3 days | 0.067 | 1.57 | 0.250 | 0.011 |
| 6 | 0 | 3 days | 2.195 | 2.09 | | |
| | 0.5 | 3 days | 0.476 | 2.41 | | |
| | 5 | 3 days | 0.023 | 1.11 | | |
| 7 | 0 | 3 days | 2.350 | 2.05 | | |
| | 0.5 | 3 days | 0.722 | 2.11 | | |
| | 5 | 3 days | 0.102 | 0.90 | | |
| 8 | 0 | 6 days | 0.508 | 1.57 | 0.178 | 0.071 |
| | 0.5 | 3 days | 0.066 | 1.00 | 0.127 | 0.081 |
| | 5 | 3 days | 0.012 | 0.53 | 0.191 | 0.054 |
| 9 | 0 | 2 weeks | 0.276 | 3.32 | 0.063 | 0.027 |
| | 5 | 2 weeks | 0.004 | 0.30 | 0.166 | 0.042 |
| 10 | 0 | 2 weeks | 0.112 | 1.87 | | |
| | 5 | 2 weeks | 0.002 | 0.29 | | |
| 11 | 0 | 9 days | 0.690 | 1.51 | | |
| | 0.5 | 9 days | 0.153 | 1.26 | | |
| | 5 | 9 days | 0.010 | 0.35 | | |

same rat. As indicated in Table IV, cholesterol feeding decreased the conversion of mevalonic acid to cholesterol by a factor of from 1.3 to 11; (average 3.9). Although the effect was consistently present, it is clear that, when compared with the inhibition by a factor of 20 to over 100 (average 65) in the conversion of acetate to cholesterol, the decrease in conversion of mevalonic acid to cholesterol cannot account for the overall depression in cholesterol synthesis which is produced by dietary cholesterol.

The apparent discrepancy between the results obtained in the squalene → cholesterol and mevalonate → squalene experiments as compared with the overall mevalonate → cholesterol experiment remains to be explained. It seems likely that the site of the relatively small inhibition observed in the mevalonate → cholesterol reactions is actually between squalene and cholesterol. The rate of the mevalonate → squalene reactions was consistently elevated by cholesterol feeding whereas the squalene → cholesterol steps were much more variably affected. The latter variations could well be due to slight differences in solubilization of squalene in the aqueous media, and this technical difficulty might then prevent the detection of a minor depression in the squalene → cholesterol portion of the pathway.

The small intestine is known to constitute a second major site of cholesterol synthesis in the body and for this reason the influence of cholesterol feeding on cholesterol synthesis from both acetate and mevalonic acid was examined in this tissue. The intestines of rats fed the cholesterol-free diet were found to carry out the synthesis of cholesterol from acetate at a rate which was one-

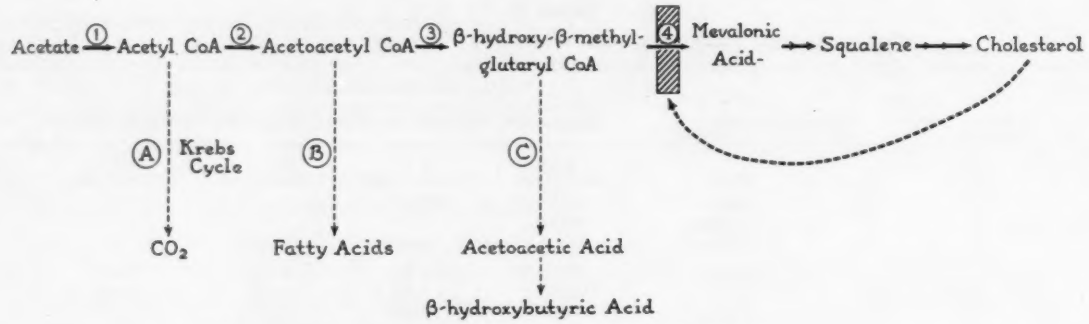



FIG. 2. PROPOSED SITE OF FEEDBACK CONTROL OF CHOLESTEROGENESIS.

half to one twenty-fifth that of liver; however, as has also been noted by Gould and associates (4), this organ proved to be immune to cholesterol feeding. Whereas the livers from rats fed 0.5 or 2.5 per cent cholesterol exhibited a marked depression in cholesterol synthesis, the intestinal slices from the same animals synthesized cholesterol as well as did the intestines from animals fed the cholesterol-free diet. As a result, a comparison of the abilities of the livers and intestines of the cholesterol-fed rats to synthesize cholesterol from acetate shows that under these circumstances much more cholesterol is produced in the *intestine* than in the liver.

The last column in Table IV presents data on the conversion of mevalonic acid to cholesterol by the intestine. As expected, this process too was unaffected by cholesterol feeding; however, the surprising aspect of this experiment is that, in contrast to the case of liver, mevalonate with two exceptions proved to be inferior to acetate as a precursor of cholesterol. In the 14 experiments, intestinal cholesterol synthesis from acetate averaged 0.17 per cent and that from mevalonic acid 0.069 per cent. It is possible that the biochemical pathway of cholesterol synthesis in the intestine may differ from that in liver, as appears to be the case in brain (21); however, these data may also reflect differences in cellular permeability for the two substrates.

### *Specific localization of the site of cholesterol inhibition*

The results of the previous experiments would indicate that the major site of the cholesterol-induced inhibition of cholesterogenesis must lie in one of the reactions before the utilization of mevalonic acid. As is shown in detail in Figure 2, four

TABLE V

*Effect of cholesterol feeding on fatty acid and ketone body synthesis* *

| Exp. | Cholesterol in diet | | Acetate-$C^{14}$ converted to: | | | |
|---|---|---|---|---|---|---|
| | | | $CO_2$ | Fatty acids | Acetoacetic acid | β-Hydroxybutyric acid |
| | % | *days* | *% added* $C^{14}$ | *% added* $C^{14}$ | *cpm*† | *cpm*† |
| 1 | 0 | 7 | 36.71 | 0.789 | 4,331 | 1,759 |
| | 2.5 | 7 | 40.94 | 0.455 | 5,126 | 1,529 |
| 2 | 0 | 20 | 18.57 | 1.623 | 3,274 | 2,284 |
| | 5 | 20 | 16.11 | 0.873 | 1,654 | 1,999 |
| 3 | 0 | 8 | 26.01 | 1.13 | 2,204 | 2,761 |
| | 2.5 | 8 | 26.51 | 6.93 | 803 | 2,459 |
| 4 | 0 | 9 | 9.21 | 6.99 | 1,808 | 3,208 |
| | 5 | 9 | 9.33 | 4.99 | 2,023 | 3,607 |
| 5 | 0 | 24 | 13.75 | 1.332 | 2,398 | 2,055 |
| | 5 | 24 | 16.50 | 0.479 | 2,878 | 1,721 |

* Conditions: flasks 1, 3 and 4 contained acetate-1-$C^{14}$, 0.5 μc; flasks 2 and 5 contained acetate-2-$C^{14}$.
† Total counts assayed at infinite thickness.

reactions are required for the conversion of acetate to mevalonic acid. All but one of these steps can be eliminated as the site of the cholesterol inhibition of cholesterol synthesis by the following experiments.

*Influence of cholesterol feeding on acetate oxidation to $CO_2$.* The first reaction in the conversion of acetate to mevalonic acid involves activation of acetate by coenzyme A and adenosine triphosphate to form acetyl CoA. As is indicated by dotted arrow A in Figure 2, this reaction is also required for the oxidation of acetate to carbon dioxide in the Krebs cycle. The effect of a high cholesterol diet on the conversion of acetate to $CO_2$ was therefore studied, and as demonstrated in the fourth column of Table V, $C^{14}O_2$ production from acetate-$C^{14}$ is not depressed by cholesterol feeding. The activation of acetate (Reaction 1, Figure 2) can therefore be eliminated as a possible site of the block in cholesterol synthesis.

*Cholesterol feeding and the conversion of acetate to fatty acids.* The second reaction in the synthesis of cholesterol consists of the condensation of two molecules of acetyl CoA to yield acetoacetyl CoA (Reaction 2, Figure 2). This reaction can be evaluated by determining the influence of cholesterol feeding on the synthesis of long chain fatty acids from acetate. As indicated by dotted arrow B, Figure 2, acetoacetyl CoA is an important intermediate in the synthesis of fatty acids as well as of cholesterol, and a block in Reaction 2 would therefore result in a depression in the incorporation of acetate-$C^{14}$ into fatty acids. Column 5 of Table V demonstrates that fatty acid synthesis is not significantly influenced by cholesterol feeding. Both Tomkins (6) and Gould (4) and their associates have reported similar findings. Reaction 2 likewise, therefore, can be eliminated as the site of the feedback reaction.

*Conversion of acetate to acetoacetic acid and β-hydroxybutyric acid.* Finally, Reaction 3, involving the condensation of a third molecule of acetyl CoA with acetoacetyl CoA to form β-hydroxy-β-methylglutaryl CoA, can be examined by determining the influence of cholesterol feeding on the conversion of acetate to the ketone bodies, acetoacetic acid and β-hydroxybutyric acid. Lynen and colleagues (22) have recently shown that β-hydroxy-β-methylglutaryl CoA is an obligatory intermediate in the synthesis of both of these ketone bodies. It follows therefore that any blockage of Reaction 3 would result in a decrease in the incorporation of acetate-$C^{14}$ into acetoacetic acid and β-hydroxybutyric acid.

As shown in columns 6 and 7 of Table V, cholesterol feeding has no influence on the synthesis of either acetoacetic acid or β-hydroxybutyric acid. This experiment demonstrates that cholesterol produces no inhibition of Reaction 3, and furthermore confirms the fact that Reactions 1 and 2 must be operating normally under circumstances when cholesterol synthesis is markedly inhibited by cholesterol feeding.

It would follow from these experiments, therefore, that as indicated by the block shown in Figure 2, the specific site of the cholesterol-induced inhibition of cholesterogenesis is at Reaction 4, involving the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid.

*Further studies on the site of the cholesterol block.* In Table VI are presented a series of experiments in which the influence of cholesterol feeding on the various reactions involved in cholesterol synthesis were examined in single rat livers. In general the results confirmed the conclusions of the previous experiments in which the different portions of the synthetic pathway were studied in separate animals.

Dietary cholesterol depressed cholesterol synthesis in these studies by a factor of from 10 to 300, and again this could not be related to increases in the concentration of liver cholesterol (columns 4 and 5, Table VI). A depression in the conversion of mevalonic acid to cholesterol was consistently noted following cholesterol feeding; however, once more this effect was in no case sufficient to account for the inhibition produced in the overall reaction from acetate to cholesterol. We would suggest, therefore, that this decrease in the conversion of mevalonic acid to cholesterol is a secondary result—perhaps due to a "disuse" depression of enzyme synthesis—of cholesterol inhibition at a site located prior to the utilization of mevalonic acid.

As was previously observed, cholesterol feeding caused an increase in the incorporation of mevalonic acid-$C^{14}$ into squalene in each of the eight experiments. In view of the postulated

TABLE VI

*Influence of cholesterol feeding on reactions related to cholesterol synthesis*

| Exp. | Cholesterol in diet | | Cholesterol concentration in liver | Cholesterol from | | Squalene from mevalonate-2-$C^{14}$ | $CO_2$ from | | Fatty acid from acetate-2-$C^{14}$ | Acetate-2-$C^{14}$ to | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Acetate-2-$C^{14}$ | Mevalonate-2-$C^{14}$ | | Acetate-2-$C^{14}$ | Mevalonate-2-$C^{14}$ | | Acetoacetate | β-Hydroxybutyrate |
| | % | *Duration* | *mg/100 g* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* | % $C^{14}$ *incorporated* | *cpm* | *cpm* |
| 1 | 0 | 8 days | 492 | 0.390 | 7.17 | 2.09 | 23.02 | 2.82 | 1.39 | 1,492 | 2,446 |
| | 2.5 | 8 days | 470 | 0.017 | 1.51 | 8.30 | 25.27 | 1.47 | 2.01 | 1,278 | 2,257 |
| 2 | 0 | 9 days | 191 | 0.434 | 12.09 | 0.34 | 5.85 | 3.09 | 0.58 | 644 | 788 |
| | 5 | 9 days | 150 | 0.002 | 0.81 | 1.12 | 3.82 | 0.78 | 0.27 | 759 | 930 |
| 3 | 0 | 9 days | 307 | 2.460 | 13.66 | 3.29 | 17.80 | 3.43 | 7.90 | 2,220 | 1,783 |
| | 5 | 9 days | 383 | 0.144 | 1.96 | 3.39 | 14.86 | 1.40 | 18.19 | 1,217 | 2,775 |
| 4 | 0 | 9 days | 182 | 0.600 | 13.64 | 0.44 | 4.42 | 4.62 | 1.89 | 1,173 | 834 |
| | 5 | 9 days | 269 | 0.002 | 1.66 | 1.58 | 3.69 | 1.48 | 0.12 | 1,023 | 1,101 |
| 5 | 0 | 11 days | 350 | 1.714 | 10.50 | 2.44 | 18.87 | 3.30 | 1.40 | 1,764 | 1,295 |
| | 2.5 | 11 days | 400 | 0.054 | 6.08 | 7.59 | 18.31 | 2.41 | 0.49 | 1,762 | 2,137 |
| 6 | 0 | 11 days | 352 | 1.367 | 7.98 | 4.67 | 16.97 | 2.98 | 1.29 | 1,296 | 1,797 |
| | 2.5 | 11 days | 383 | 0.053 | 0.66 | 10.13 | 14.40 | 0.86 | 4.17 | 1,745 | 2,818 |
| 7 | 0 | 11 days | 217 | 0.866 | 4.44 | 3.18 | 14.57 | 1.72 | 5.16 | 2,447 | 1,367 |
| | 2.5 | 11 days | 211 | 0.048 | 1.48 | 5.01 | 16.38 | 1.07 | 4.60 | 2,164 | 1,369 |
| 8 | 0 | 11 days | 189 | 0.646 | 4.74 | 1.24 | 7.98 | 1.44 | 3.13 | 2,178 | 886 |
| | 2.5 | 11 days | 208 | 0.061 | 1.38 | 5.78 | 10.35 | 0.73 | 3.82 | 2,363 | 1,383 |

block shown in Figure 2, it seems likely that this effect of dietary cholesterol on the conversion of $C^{14}$ mevalonate to squalene may be due to differential isotopic dilution of the mevalonate. An inhibition of mevalonic acid synthesis would lead to a decrease in the size of the mevalonic acid pool, and the dilution of the added radioactive mevalonate would therefore be less in the livers of the cholesterol-fed rats. The resulting greater specific activity of the mevalonate utilized for squalene synthesis would then cause an increased incorporation of $C^{14}$ into squalene.

Finally, the results shown in Table VI again demonstrate that the conversion of acetate to $CO_2$, fatty acids and ketone bodies is not significantly influenced by dietary cholesterol. These studies serve to confirm the conclusion that the primary site of the cholesterol feedback control must be located at the point of conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid.

It is noteworthy that mevalonic acid-2-$C^{14}$ was converted to $C^{14}O_2$ at significant rates in all of the experiments presented in Table VI. Since the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid has not been found to be reversible (20, 23), it is unlikely that this $C^{14}O_2$ is derived from the conversion of mevalonate to acetyl CoA with subsequent oxidation of the latter in the Krebs cycle. As shown by Cornforth, Cornforth, Popják and Gore (24), the second carbon of mevalonate contributes to six carbons, including two of the angular methyl groups, of lanosterol, and one of these labeled methyl groups must be oxidixed to $CO_2$ during the conversion of lanosterol to cholesterol. It follows, therefore, that for each carbon-2 of mevalonic acid converted to $CO_2$, five should be incorporated into cholesterol. The observed values for the ratio: ($C^{14}O_2$ from mevalonate-2-$C^{14}$) : (cholesterol-$C^{14}$ from mevalonate-2-$C^{14}$) in the normal animals varied from 1 : 2.5 to 1 : 4.0 and averaged 1 : 3.2. In view of the possibility that precursors of cholesterol, which have lost one or two of their angular methyl carbons, may also accumulate in this system, the observed ratios are consistent with the concept that most, i.e., approximately two-thirds, and perhaps all of the $C^{14}O_2$ produced from mevalonate-2-$C^{14}$, is a consequence of mevalonate conversion to sterols

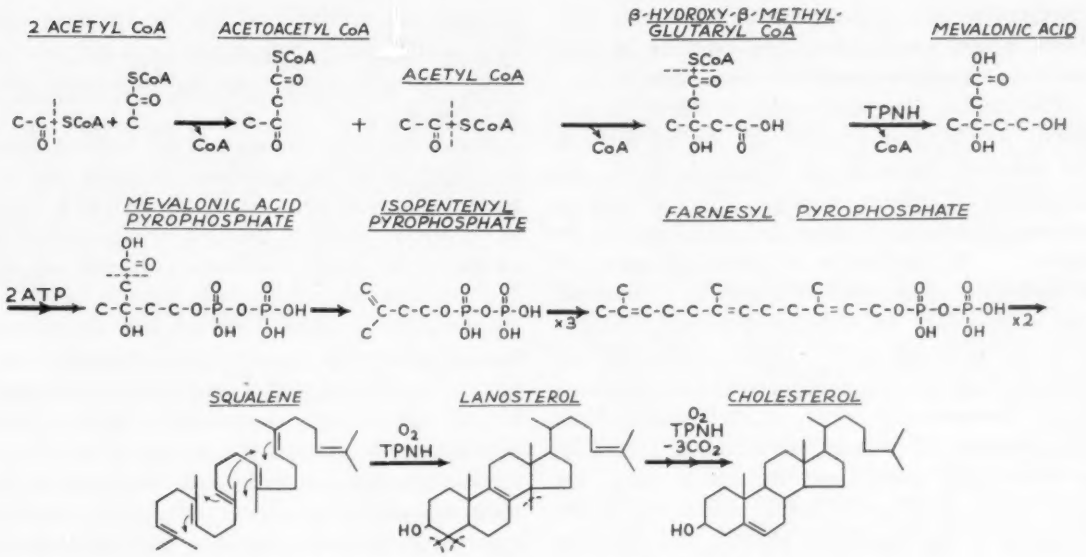



FIG. 3. PATHWAY OF CHOLESTEROL SYNTHESIS.

rather than of breakdown to acetyl CoA. This conclusion is further supported by the finding that less $C^{14}O_2$ is produced from mevalonate-2-$C^{14}$ when the conversion of mevalonate to cholesterol is somewhat suppressed by cholesterol feeding.

## DISCUSSION

The present study represents an attempt to elucidate the mechanism by which cholesterol feeding is able to inhibit cholesterol synthesis within the liver cell. The negative feedback reaction involved in this phenomenon must constitute an extremely sensitive homeostatic regulator of cholesterol concentration since, as demonstrated here (Table I), an inhibition of cholesterol synthesis by a factor of at least 100 can take place before any increase in cholesterol concentration detectable by current analytical procedures has occurred. Such a process is obviously well suited both to counteract minor variations in dietary cholesterol and to mediate the self regulation of *endogenous* cholesterol synthesis.

The major biochemical steps now believed to be involved in the synthesis of cholesterol are summarized in Figure 3. The most obvious mechanism by which dietary cholesterol might depress the rate of such a series of reactions is through a mass-action effect on the last reaction of the series. Two pieces of evidence, however, argue against this being *the* important mechanism by which cholesterol inhibits its own synthesis. First, marked depression of cholesterol synthesis takes place in the absence of any significant increase in the concentration of cholesterol (Tables I and VI). Second, the conversion of squalene to cholesterol is not sufficiently depressed by cholesterol feeding to account for the observed decrease in overall cholesterol synthesis (Table II). Since the final step in cholesterol synthesis is included in these reactions, a mass-action effect on this terminal reaction cannot be the *major* means by which cholesterol depresses cholesterol synthesis. With the further observation (Table III) that cholesterol feeding results in an increased rate of conversion of mevalonic acid to squalene, it is apparent that the control mechanism for cholesterol synthesis must actually be located in the initial steps of cholesterogenesis. A similar conclusion has been drawn by Gould and Popják (11) and by Bucher and associates (12).

The series of studies reported here has further led to the conclusion that the specific location of the site of regulation of cholesterol synthesis is at the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid (Figure 2, Tables V and VI). A second less effective site of feedback inhibition may exist in the reactions between squalene and cholesterol. The latter effects cannot, however, account for the overall inhibition of cholesterol synthesis from acetate and may well

represent a secondary depression of enzyme synthesis which would follow the decrease in cholesterol precursors caused by the earlier block.

The finding that cholesterol synthesis is primarily regulated at a single site located early in the reaction sequence is significant from the standpoint of cellular economy. Each of the reactions prior to the synthesis of mevalonate is involved in the production of other end products as well as of cholesterol (Figure 2); in contrast, the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid represents the first reaction which is unique to cholesterol synthesis in the conversion of acetate to cholesterol. Since this reaction is probably irreversible (20, 23), a controlling mechanism located at any site later in the reaction sequence would lead to the trapping of intermediates which could probably not be utilized. On the other hand, a feedback regulator located prior to the synthesis of β-hydroxy-β-methylglutaryl CoA, while less wasteful, would result in an incidental depression of the synthesis of fatty acids and ketone bodies whenever cholesterol synthesis was decreased. The location of the controlling reaction immediately following the last "branch point" of the chain of reactions leading to cholesterol synthesis is therefore best suited to regulate this overall synthesis with a minimum of secondary biochemical disturbances.

Although certain instances of products inhibiting their own synthesis are known in higher animals, the specific depression of one of a series of reactions by the final product of these reactions has not been previously demonstrated in animal tissues. This type of control mechanism is, however, quite well known in bacteria. In 1950, Gots first noted that the addition of purines to a culture of *Escherichia coli* inhibited the synthesis of the purine precursor, 5-amino-4-imidazolecarboxamide (25). Subsequently, Adelberg and Umbarger observed that exogenous valine depressed the formation of its precursor, β-ketoisovaleric acid (26). Pyrimidine synthesis is also controlled in *E. coli* by such a mechanism, since both uracil and cytosine will specifically inhibit the synthesis of ureidosuccinic acid (27). In bacteria, such feedback inhibition has been shown to be the result of end products inhibiting enzymatic reactions either directly (28, 29) or by suppressing the synthesis of specific enzymes (30, 31). Furthermore, both mechanisms may operate in the same organism to suppress the same reaction (32, 33).

It is noteworthy that each of the bacterial feedback systems so far described possesses two important characteristics: first, the feedback regulation always involves the first reaction which is unique to the specific synthetic sequence, i.e., the first reaction beyond the last "branch point" of the metabolic pathway. Second, this site of control in the bacterial systems is an irreversible reaction. Our finding that a feedback system manifesting both of these characteristics is present in higher animals suggests that a very similar, if not identical, type of specific negative feedback mechanism may constitute a more widespread means of controlling metabolic reactions than has hitherto been realized.

Support for this concept is provided by the existence in higher animals of several known examples in which the feeding of a compound suppresses the synthesis of this compound. Purine synthesis is depressed by purine feeding in the rat (34, 35). Wyngaarden, Silberman, Sadler and Ashton, using pigeon liver, have extensively studied the *in vitro* effects of purine derivatives on the reactions involved in their synthesis (36–38). While the site at which the normal control of purine synthesis occurs has not been established, several purine nucleotides have been shown to inhibit the enzymes involved in early steps of this process, and a specific feedback mechanism similar to that in bacteria has therefore been postulated to explain these observations. Tyrosine feeding depresses the hydroxylation of phenylalanine to tyrosine in HeLa cell structures (39). Since the conversion of phenylalanine to tyrosine involves only one enzymatic step, it is not necesary to invoke a feedback control to a distant reaction site to account for inhibition; nonetheless, it has been shown that the activity, and probably the synthesis of the single enzyme involved, is depressed by tyrosine feeding (39, 40). Finally, although the control mechanism has not been investigated, lipogenesis is inhibited in rats by dietary fat (41).

The demonstration that feedback control of an enzyme far removed from the end product can occur in mammalian tissues raises the question of the role that this mechanism may play in pathological

conditions. One might speculate that failure of this type of feedback mechanism, due perhaps to a genetically-derived insensitivity of the single crucial enzyme to its inhibitor, would lead to an excessive synthesis of the end product. According to this concept, familial hypercholesterolemia could be the result of such a defect in the enzyme converting β-hydroxy-β-methylglutarate to mevalonate.

## SUMMARY

1. The mechanism by which dietary cholesterol inhibits the synthesis of cholesterol from acetate has been studied in rat liver slices.

2. Evidence is presented which indicates that the primary site of this feedback regulation is at the first reaction on the pathway of cholesterol synthesis which is unique to this synthesis, that is, at the conversion of β-hydroxy-β-methylglutaryl CoA to mevalonic acid.

3. The characteristics of this feedback control are discussed and it is concluded that the regulation of cholesterol synthesis involves a negative feedback system possessing characterisics very similar to those that have previously been described for bacteria.

## ACKNOWLEDGMENT

The authors wish to thank Miss Deirdre V. LaMarr for her technical assistance in these studies.

## REFERENCES

1. Siperstein, M. D., and Guest, M. J. Studies on the homeostatic control of cholesterol synthesis (abstract). J. clin. Invest. 1959, **38**, 1043.
2. Siperstein, M. D., and Guest, M. J. The feed-back control of cholesterol synthesis (abstract). Amer. J. Med. 1959, **27**, 325.
3. Gould, R. G. Lipid metabolism and atherosclerosis. Amer. J. Med. 1951, **11**, 209.
4. Gould, R. G., Taylor, C. B., Hagerman, J. S., Warner, I., and Campbell, D. J. Cholesterol metabolism. I. Effect of dietary cholesterol on the synthesis of cholesterol in dog tissue in vitro. J. biol. Chem. 1953, **201**, 519.
5. Gould, R. G., and Taylor, C. B. Effect of dietary cholesterol on hepatic cholesterol synthesis. Fed. Proc. 1950, **9**, 179.
6. Tomkins, G. M., Sheppard, H., and Chaikoff, I. L. Cholesterol synthesis by liver. III. Its regulation by ingested cholesterol. J. biol. Chem. 1953, **201**, 137.
7. Frantz, I. D., Jr., Schneider, H. S., and Hinkelman, B. T. Suppression of hepatic cholesterol synthesis in the rat by cholesterol feeding. J. biol. Chem. 1954, **206**, 465.
8. Harper, P. V., Jr., Neal, W. B., Jr., and Hlavacek, G. R. Lipid synthesis and transport in the dog. Metabolism 1953, **2**, 69.
9. Hotta, S., and Chaikoff, I. L. The role of the liver in the turnover of plasma cholesterol. Arch. Biochem. 1955, **56**, 28.
10. Popják, G., and Beeckmans, M. L. Extrahepatic lipid synthesis. Biochem. J. 1950, **47**, 233.
11. Gould, R. G., and Popják, G. Biosynthesis of cholesterol *in vivo* and *in vitro* from DL-β-hydroxy-β-methyl-δ-[2-$^{14}$C]-valerolactone. Biochem. J. 1957, **66**, 51P.
12. Bucher, N. L. R., McGarrahan, K., Gould, E., and Loud, A. V. Cholesterol biosynthesis in preparations of liver from normal, fasting, X-irradiated, cholesterol-fed, triton, or $\Delta^4$-cholesten-3-one-treated rats. J. biol. Chem. 1959, **234**, 262.
13. Bucher, N. L. R. Alterations of cholesterol biosynthesis in liver cell fractions from rats in various experimental conditions *in* Biosynthesis of Terpenes and Sterols, Ciba Foundation Symposium on Biosynthesis of Terpenes and Sterols, G. E. W. Wolstenholme and M. O'Connor, Eds. 1959, p. 46.
14. Siperstein, M. D., and Fagan, V. M. Studies on the relationship between glucose oxidation and intermediary metabolism. I. The influence of glycolysis on the synthesis of cholesterol and fatty acid in normal liver. J. clin. Invest. 1958, **37**, 1185.
15. Heilbron, I. M., Kamm, E. D., and Owens, W. M. The unsaponifiable matter from the oils of elasmobranch fish. Part I. A contribution to the study of the constitution of squalene (spinacene). J. chem. Soc. 1926, Part I, 1630.
16. Sperry, W. M., and Webb, M. A revision of the Schoenheimer-Sperry method for cholesterol determination. J. biol. Chem. 1950, **187**, 97.
17. Van Slyke, D. D. Studies of acidosis. VII. The determination of β-hydroxy-butyric acid, acetoacetic acid, and acetone in urine. J. biol. Chem. 1917, **32**, 455.
18. Olson, J. A., Jr., Lindberg, M., and Bloch, K. On the demethylation of lanosterol to cholesterol. J. biol. Chem. 1957, **226**, 941.
19. Lynen, F., Eggerer, H., Henning, U., and Kessel, I. Farnesyl-pyrophosphat und 3-methyl-$\Delta^3$-butenyl-1-pyrophosphate, die biologischen Vorstufen des Squalens. III. Zur Biosynthese der Terpene. Angew. Chem. 1958, **70**, 738.
20. Rilling, H., Tchen, T. T., and Bloch, K. On the mechanism of squalene biogenesis. Proc. nat. Acad. Sci. 1958, **44**, 167.
21. Grossi, E., Paoletti, P., and Paoletti, R. An analysis of brain cholesterol and fatty acid biosynthesis. Arch. int. Physiol. 1958, **66**, 564.

22. Lynen, F., Henning, U., Bublitz, C., Sörbo, B., and Kröplin-Rueff, L. Der chemische Mechanismus der Acetessigsäurebildung in der Leber. Biochem. Z. 1958, **330**, 269.
23. Siperstein, M. D. Unpublished observations.
24. Cornforth, J. W., Cornforth, R. H., Popják, G., and Gore, I. Y. Studies on the biosynthesis of cholesterol. 5. Biosynthesis of squalene from DL-3-hydroxy-3-methyl-[2-$^{14}$C] pentano-5-lactone. Biochem. J. 1958, **69**, 146.
25. Gots, J. S. Accumulation of 5(4)-amino 4(5)-imidazolecarboxamide in relation to sulfonamide bacteriostasis and purine metabolism in *Escherichia coli*. Fed. Proc. 1950, **9**, 178.
26. Adelberg, E. A., and Umbarger, H. E. Isoleucine and valine metabolism in *Escherichia coli*. V. A ketoisovaleric acid accumulation. J. biol. Chem. 1953, **205**, 475.
27. Yates, R. A., and Pardee, A. B. Control of pyrimidine biosynthesis in *Escherichia coli* by a feedback mechanism. J. biol. Chem. 1956, **221**, 757.
28. Brooke, M. S., Ushiba, D., and Magasanik, B. Some factors affecting the excretion of orotic acid by mutants of Aerobacter aerogenes. J. Bact. 1954, **68**, 534.
29. Gots, J. S. Purine metabolism in bacteria. V. Feedback inhibition. J. biol. Chem. 1957, **228**, 57.
30. Vogel, H. J. Repression and induction as control mechanisms of enzyme biogenesis: The "adaptive" formation of acetylernithinase *in* The Chemical Basis of Heredity, W. D. McElroy and B. Glass, Eds. Baltimore, The Johns Hopkins Press, 1957, p. 276.
31. Umbarger, H. E. Evidence for a negative-feedback mechanism in the biosynthesis of isoleucine. Science 1956, **123**, 848.
32. Yates, R. A., and Pardee, A. B. Control by uracil of formation of enzymes required for orotate synthesis. J. biol. Chem. 1957, **227**, 677.
33. Umbarger, H. E., and Brown, B. Isoleucine and valine metabolism in *Escherichia coli*. VII. A negative feedback mechanism controlling isoleucine biosynthesis. J. biol. Chem. 1958, **233**, 415.
34. Abrams, Richard. Some factors influencing nucleic acid purine renewal in the rat. Arch. Biochem. 1951, **33**, 436.
35. Goldthwait, D. A., and Bendich, A. Effects of a folic acid antagonist on nucleic acid metabolism. J. biol. Chem. 1952, **196**, 841.
36. Wyngaarden, J. B., Silberman, H. R., and Sadler, J. H. Feedback mechanisms influencing purine ribotide synthesis. Ann. N. Y. Acad. Sci. 1958, **75**, 45.
37. Wyngaarden, J. B., and Ashton, D. M. Feedback control of purine biosynthesis by purine ribonucleotides. Nature (Lond.) 1959, **183**, 747.
38. Wyngaarden, J. B., and Ashton, D. M. The regulation of activity of phosphoribosylpyrophosphate amidotransferase by purine ribonucleotides: A potential feedback control of purine biosynthesis. J. biol. Chem. 1959, **234**, 1492.
39. Eagle, H., Piez, K. A., and Fleischman, R. The utilization of phenylalanine and tyrosine for protein synthesis by human cells in tissue culture. J. biol. Chem. 1957, **228**, 847.
40. Auerbach, V. H., and Waisman, H. A. The metabolism of aromatic amino acids in leukemic rats. Cancer Res. 1958, **18**, 536.
41. Hill, R., Linazasoro, J. M., Chevallier, F., and Chaikoff, I. L. Regulation of hepatic lipogenesis: The influence of dietary fats. J. biol. Chem. 1958, **233**, 305.

# GASTRODIALYSIS IN THE TREATMENT OF ACUTE RENAL FAILURE * †

By THOMAS A. MARR, JAMES M. BURNELL AND BELDING H. SCRIBNER WITH THE TECHNICAL ASSISTANCE OF W. R. KORESKI

*(From the Veterans Administration Hospital and the Department of Medicine, University of Washington, Seattle, Wash.)* ‡

(Submitted for publication September 8, 1959; accepted December 10, 1959)

The objectives of dialysis in the management of acute renal failure include both correction and prevention of various electrolyte disorders and removal of nitrogenous products responsible for the uremic syndrome.

In the past, gastric lavage has failed as a method of dialysis because of uncontrollable electrolyte transfers and loss of large amounts of dialysis fluid to the patient. The use of a cellophane bag, suggested originally by Schloerb (1, 2), has solved these problems and has made gastrodialysis technically possible. The theoretical advantage of a method of continuous dialysis as compared with intermittent dialysis offers great appeal in that it might be possible to obviate the major fluctuations in the uremic state inherent in intermittent dialysis. Further, if continuous dialysis were sufficiently effective in removing metabolic wastes, as well as in correcting electrolyte abnormalities, it might be possible to avoid the costly, time-consuming, highly technical procedure of hemodialysis or significantly decrease the number of hemodialyses necessary to sustain the patient through a period of renal failure.

The present study was undertaken to evaluate the use of dialysis of the stomach as an adjunct in management of patients with acute renal failure. This paper describes a technique of gastrodialysis and the evaluation of this procedure in the treatment of twelve adults and two children.

## THE TECHNIQUE OF GASTRODIALYSIS

Gastrodialysis is carried out through a cellophane bag which is tied to the end of a nasogastric tube and swallowed. Construction of a bag from a flat sheet of cellophane is shown in Figure 1. Although any no. 14 French plastic gastric tube may be used, we prefer, because it is stiff when cold, a no. 14 French Bardic tube.[1] This tube is cut about 6 inches from the end which is to be inserted. A one inch length of glass tubing that will fit tightly inside the plastic tubing is used to rejoin the two pieces of tubing (Figure 1a). This joint is wrapped tightly with surgical silk. The tip is cut off a small rubber balloon; the balloon is then slipped over the joint (Figure 1b). A cellophane bag is made from a 24 by 25 inch sheet of plain, non-waterproof cellophane (PT 300)[2] by wetting the sheet and gathering all the edges around the plastic tube. The cellophane bag is tied over the glass joint and rubber with a single, firm tie which is placed near the end of the glass tube away from the bag (Figure 1c). The edges of cellophane are cut close to the single tie (Figure 1c) and the rubber folded over the top of the cellophane bag. Two more ties are placed (Figure 1d). The rubber is then folded back over these last ties to protect the esophagus from abrasion (not shown in Figure 1). The final volume of bags made in this manner will be 1,000 to 1,200 ml. The larger bags provide more surface area for dialysis.

Insertion of the bag into the stomach is accomplished using a technique similar to that used in passing a stomach tube. Because the bag is bulky, it is necessary to pass the tube through the mouth. It is advisable to anesthetize the pharynx. Anethesia is accomplished with 0.25 per cent pontocaine or 5 per cent Cyclaine in a spray atomizer. Care is taken not to anesthetize the vocal cords. With the patient's head tilted slightly forward, the cooled bag is then pushed into the esophagus. After this maneuver the patient can swallow, permitting the bag to enter the stomach. The tube then can be threaded retrograde through the nose if desired. Once in place, the bag causes a minimum of discomfort even when full.

Dialysis fluid is placed in the bag and changed every 20 minutes thereafter. It is advisable to start with a 200 to 300 ml cycle which is then gradually increased to 600 to 800 ml over a period of a few hours. This procedure permits the patient to become accustomed to the

* Presented at the meeting of the American Federation for Clinical Research, Atlantic City, N. J., May, 1958.

† Supported by Abbott Laboratories; the National Institutes of Health, Grant 6767; The Schweppe Foundation; the Washington State Heart Association; and the Washington State Fund for Biology and Medicine.

‡ Present address of the authors: Department of Medicine, University of Washington, Seattle 5, Wash.

[1] Manufactured as item No. 1005 by C. R. Bard, Summit, N. J.

[2] A thinner cellophane is manufactured by J. P. Bemberg Co., Wuppertal-Barmer, Germany. This cellophane is less bulky and appears to permit more efficient dialysis.

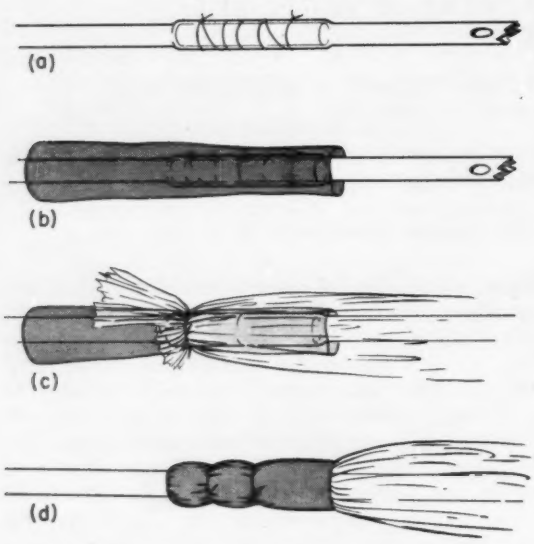



FIG. 1. CONSTRUCTION OF CELLOPHANE GASTRODIALYSIS BAG. See text for details.

rapid filling and emptying of his stomach. The dialysis is usually continued 24 hours per day until renal function improves.

Cycling can be accomplished manually by attaching the gastric tube through a "T" tube to a reservoir above the bed and a drain below. Clamps or glass stopcocks on the two branches control the flow in from the reservoir and out into the drain. Drainage of the bag can be hastened by suction.

As manual filling and emptying of the bag requires nearly the full time service of an attendant, an automatic cycling apparatus has been devised that permits continuous dialysis with only occasional attention. This apparatus automatically heats and cycles any preselected volume of dialysis fluid in and out of the cellophane bag (Figure 2).[3]

It is advisable to warm the dialysis fluid to body temperature to avoid the patient's expending his own calories to heat the dialysate.

## MATERIALS AND METHODS

In 14 patients with acute renal failure gastrodialysis was performed for varying lengths of time ranging from 2 to 16 days, with a mean of 6 days. Data were obtained in 98 balance periods ranging from 20 minutes to 24 hours, but averaging 12 hours.

Dialysis fluid was compounded in 20 L volumes by addition of previously weighed glucose, NaCl, and $NaHCO_3$. Nonprotein nitrogen in the dialysate was determined using a modified micro-Kjeldahl method (3) and subsequent Nesslerization (4). Nonprotein nitrogen in blood and urine was determined by the Nesslerization method (4). Sodium and potassium were determined by Baird flame photometry using an internal standard. Chloride (5), bicarbonate (6), and titratable acid (7) were performed by titration methods. Glucose analysis in the dialysate was performed by vacuo-drying (8) with correction for other salts. Blood glucose was measured by the method of Benedict (9). Water transfer was determined by the total change of weight of dialysate in patients in whom there was no significant sodium transfer.

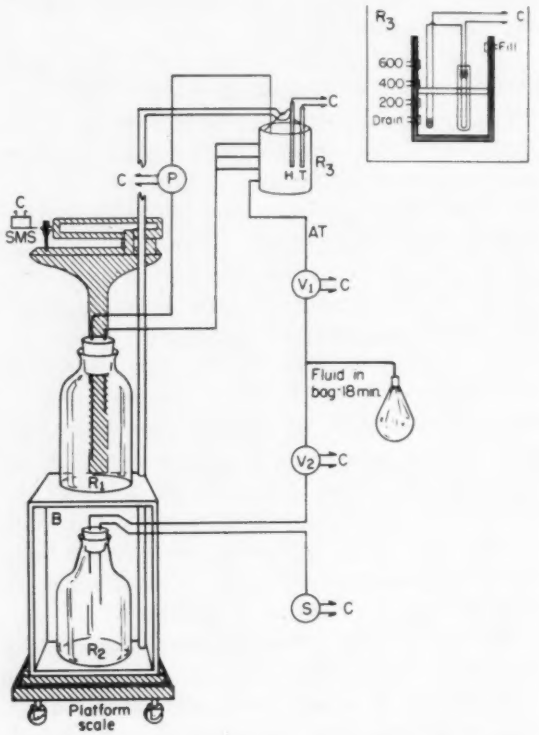



FIG. 2. SCHEMATIC DRAWING OF CYCLING APPARATUS. $R_1$: 20 L glass or plastic carboy containing fresh dialysate which is pumped by pump P to reservoir $R_3$. Cross-section of $R_3$ in cut at upper right. Fluid is warmed or cooled as desired here and flows by gravity into bag. $V_1$ and $V_2$ are solenoid valves that control inflow and outflow, respectively, of the bag. Fluid is pumped from bag to reservoir $R_2$ by vacuum created by pump S. The entire apparatus is controlled by clock C, which activates the various components at the correct time. Should the bag break and the patient begin to absorb dialysate, the loss of water from the system moves the scale. This activates SMS, a safety micro-switch, which turns off the clock, starts gastric suction, and turns on the nurse's call light.

[3] Detailed plans of the automatic cycling device or the completed device may be obtained from Mr. Wayne Quinton, Medical Instrument Shop, Health Sciences Building, University of Washington, Seattle 5, Wash.

TABLE I

*Sodium and chloride transfer during dialysis*

| Sodium dialysate composition | Patients | Mean duration dialysis | Mean cycle vol | Transfer per 24 hours | |
|---|---|---|---|---|---|
| | | | | Range | Mean |
| | *no.* | *hrs* | *ml* | *mEq* | *mEq* |
| No electrolyte | 6 | 51 | 340 | −80 to −190 | −115 |
| 50 mEq Na/L | 4 | 59 | 400 | 0 to −70 | −17 |
| 100 mEq Na/L | 4 | 176 | 233 | 10 to 192 | +90 |
| Chloride | | | | | |
| 0 mEq $Cl^-$ | 3 | 72 | 370 | −139 to −275 | −224 |
| 50 mEq $Cl^-$ | 2 | 70 | 320 | −192 to +16 | −60 |
| 100 mEq $Cl^-$ | 4 | 93 | 230 | −114 to +54 | +12 |

## RESULTS

*Magnitude of solute transfers.* The magnitude of solute transfers is a function of many variables. These include membrane characteristics, temperature, time, volume, and chemical constitution of gastric secretions and dialysis fluid. In these studies, PT-300 wettable cellophane was used throughout. The temperature of the dialysate was elevated to 37° C. A cycle time of 20 minutes was selected after preliminary studies showed a rapid decrease in nitrogen after this period. The transfer data were obtained at cycle volumes ranging from 150 to 700 ml per cycle. The capacity of the gastrodialysis bags ranged from 1,000 to 1,500 ml.

Results of gastrodialysis can be considered in two general areas: *1*) correction and prevention of various electrolyte disorders; and *2*) nitrogen removal and prevention of uremia.

*Sodium transfer.* Transfer data were obtained using dialysate with sodium concentrations of 0, 50, and 100 mEq per L. The data summarized in Table I suggest that the concentration of sodium in the dialysis fluid for zero net transfer of sodium was about 50 mEq per L. Concentrations above this figure added sodium to the patient, while absence of sodium resulted in removal of sodium from the patient.

In six patients no sodium was added to the dialysate. The mean duration of dialysis was 51 hours. The mean cycle volume was 340 ml per cycle, or slightly greater than 1 L per hour. Removal of sodium ranged from 80 to 190 mEq per 24 hours with a mean removal of 115 mEq per 24 hours. In four patients, a dialysate sodium concentration of 50 mEq per L resulted in sodium removal ranging from 0 to 70 mEq per 24 hours; mean removal was 17 mEq per 24 hours. In four patients dialysis with a fluid containing 100 mEq per L of sodium resulted in the addition of from 15 to 192 mEq of sodium per 24 hours. The mean net transfer to each patient was 90 mEq per 24 hours.

*Chloride transfer.* Chloride transfer was studied in three patients (Table I) dialyzed with no chloride in the dialysate. The removal rate varied from 139 to 275 mEq per 24 hours; the mean was 224 mEq per 24 hours. At times it became necessary to limit or to prevent chloride removal. In two patients studied at dialysate concentrations of 50 mEq per L, the removal averaged 60 mEq per 24 hours. In four patients dialyzed against concentrations of chloride of 100 mEq per L, there was the addition to each patient of a mean of 12 mEq per 24 hours.

*Correction and prevention of acidosis.* Alterations of the acid-base status during gastrodialysis can take place in two ways. First, if dialysis is performed with a nonhydrogen ion-containing fluid, hydrogen ions may be removed. Second, if the dialysate contains hydrogen ion acceptors, such as bicarbonate ions, the addition of bicarbonate will buffer hydrogen ions within the body. Such transfer takes place either in exchange for chloride or as an addition with sodium ions.

Hydrogen ion transfer is summarized in Table

II. In nine patients dialyzed a mean of 33 hours, the mean hydrogen ion removal was 110 mEq per 24 hours. Bicarbonate ion transfer cannot be easily studied. If the bicarbonate is measured in the dialysate after passage through the stomach, the decrease measures the sum of the bicarbonate transferred to the patient plus hydrogen ion removal from the stomach. Without measurement of $CO_2$ gas evolved, $H^+ + HCO_3^- \rightarrow H_2CO_3 \rightarrow H_2O + CO_2\uparrow$, there is no way of measuring the amount of decrease in $HCO_3^-$ attributable to $H^+$ removal. However, the total fall in $HCO_3^-$ does measure the total alkalinizing effect on the patient. In three patients dialyzed at a $HCO_3^-$ concentration of 50 mEq per L, the mean loss from dialysate was 138 mEq per 24 hours. In two patients dialyzed at a $HCO_3^-$ concentration of 100 mEq per L, the mean $HCO_3^-$ decrease was 190 mEq per 24 hours.

In all 14 patients it was possible to effect rates of combined hydrogen ion and bicarbonate transfer without sodium intake which were sufficient to correct and prevent acidosis. If no acidosis existed, no $HCO_3^-$ was added to the dialysate. If acidosis was severe, 100 mEq per L of $HCO_3^-$ was added to the dialysate. Vigorous attempts to correct acidosis can result in very rapid exchanges. It is advisable, therefore, to monitor both the transfers in the bath and also to perform daily serum bicarbonate determinations in order to avoid metabolic alkalosis. In only one patient was it necessary to add acid to the dialysate. Addition of 50 mEq per L of HCl resulted in the transfer to the patient of 112 mEq per 24 hours of hydrogen ions.

Due to the very large potential rate of hydrogen and bicarbonate exchange, much smaller gastrodialysis bags and cycle volumes can be used, provided the sole object of gastrodialysis is correction of acidosis. This possibility is illustrated by the following case.

A 32 year old woman with acute renal failure and generalized edema suffered from acidosis out of proportion to other complications. Her serum bicarbonate was 7.5 mEq per L. A gastrodialysis bag was made from a 24 inch length of cellophane tubing one inch in diameter with a capacity of about 200 ml. One hundred ml of solution containing 50 mEq per L sodium bicarbonate in dextrose was cycled every 20 minutes for 12 hours. This resulted in a decrease in dialysate $HCO_3^-$ of 190 mEq in 12 hours and no net transfer of sodium. She received in addition 50 mEq of sodium lactate intravenously. Her serum bicarbonate rose from 7.5 to 12.5 mEq per L. Subsequently, gastrodialysis using only dextrose and water was sufficient to restore normal acid-base status balance and to remove sodium, decreasing the edema.

*Potassium removal.* This was usually desired. When potassium was omitted from the dialysis fluid, potassium was invariably removed. In six patients who were studied a mean of 82 hours each with a mean cycle volume of 280 ml, potassium removal ranged from 6 to 21 mEq per 24 hours, with a mean removal of 13 mEq per 24 hours. In six patients studied a mean of 30 hours each with a mean cycle volume of 450 ml, removal ranged from 8 to 43 mEq with a mean of 25 mEq per 24 hours. It appears likely that larger cycle volumes will be associated with removal of larger amounts of potassium.

These rates are sufficient to remove significant quantities of potassium if gastrodialysis is begun

TABLE II

*Hydrogen and bicarbonate ion transfer during dialysis*

| Hydrogen dialysate composition | Patients | Mean duration dialysis | Mean cycle vol | Transfer per 24 hours | |
|---|---|---|---|---|---|
| | | | | Range | Mean |
| | *no.* | *hrs* | *ml* | *mEq* | *mEq* |
| No HCl Added | 9 | 33 | 295 | 22–400 | −110 |
| 50 mEq/L of HCl | 1 | 36 | 330 | 96–120 | +112 |
| Bicarbonate | | | | | |
| 50 mEq/L of $HCO^-_3$ | 3 | 11.5 | 280 | 40–180 | +138 |
| 100 mEq/L of $HCO^-_3$ | 2 | 16 | 250 | | +190 |

TABLE III

*Removal of nonprotein nitrogen*

| Cycle volume | Patients | Mean duration dialysis | Mean cycle vol | Removal per 24 hours | |
|---|---|---|---|---|---|
| | | | | Range | Mean |
| *ml* | *no.* | *hrs* | *ml* | *g* | *g* |
| 200–400 | 8 | 99 | 270 | 0.6–2.5 | 2.0 |
| 400–600 | 6 | 34 | 530 | 1.1–5.6 | 3.0 |

early in the course of renal failure. Gastrodialysis will prevent serious hyperkalemia unless there is marked catabolic activity. Correction of acidosis helps ameliorate the problem of hyperkalemia since increasing pH is associated with lowering of the serum potassium level (10).

*Water removal.* The term water removal is used to connote the transfer of water free of electrolyte. Data were obtained at dialysate sodium concentrations of 50 mEq per L. Thus, since sodium transfer at this concentration was near zero, increase in the weight of the dialysate reflected removal of solute free water from the patient.

Transfer of water was controlled by the regulation of the concentration of dextrose in the dialysate. In virtually all patients, dialysis was performed using 20 per cent dextrose. The use of 40 per cent dextrose in two patients resulted in water removal of up to 1,000 ml in 24 hours. Water transfer data using 20 per cent N dextrose were obtained in six patients. In these, the duration of dialysis averaged 82 hours with mean cycle volumes of 270 ml. Water removal ranged from zero to 600 ml per 24 hours, the mean removal was 200 ml per 24 hours. As long as the dialysate concentration was hyperosmolal, there were no instances of water absorption by the patient. Thus, in patients in whom it was desirable to have a negative free water balance, the total of sensible, insensible, urinary and dialytic losses permitted a negative balance of 1 to 3 L per 24 hours.

*Glucose administration.* Glucose transfer at a 20 per cent concentration was studied in six patients dialyzed a mean of 45 hours and a mean cycle volume of 390 ml. The transfer to the patient ranged from 26 to 452 g per 24 hours with a mean of 150 g per 24 hours. If 40 per cent glucose was used, the mean addition to two patients studied was 450 g per 24 hours. In most patients, slightly elevated blood glucose concentrations were maintained. In one patient the blood glucose concentration rose to slightly more than 1,000 mg per 100 ml.

*Nonprotein nitrogen removal.* Nonprotein nitrogen removal was used as an index of the removal of substances responsible for the uremic syndrome. In eight patients studied at a mean cycle volume of 270 ml for a mean of 99 hours each, nonprotein nitrogen removal ranged from 0.6 to 2.5 g per 24 hours. The mean was 2.0 g per 24 hours (Table III).

In six patients studied at a mean cycle volume of 530 ml for a mean duration of 34 hours, the nonprotein nitrogen removal ranged from 1.1 to 5.6 g per 24 hours with a mean of 3.0 g per 24 hours.

An additional variable affecting nitrogen removal appears to be the body concentration of nitrogen. Figure 3 illustrates the influence of cycle volume in three patients having different

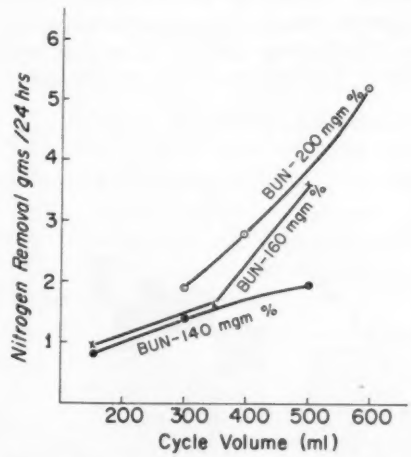



FIG. 3. THE INFLUENCE OF CYCLE VOLUME OF DIALYSIS FLUID ON NITROGEN REMOVAL IS PLOTTED FOR THREE PATIENTS AT DIFFERENT ELEVATIONS OF BLOOD UREA NITROGEN.

concentrations of urea nitrogen. It can be seen that not only does nitrogen yield increase as cycle volume increases, but also as the blood urea nitrogen increases.

The use of a small number of patients to evaluate the efficacy of gastrodialysis in the prevention of the uremic syndrome is fraught with hazard. The individual variation in the course of the disease is too great to permit definite conclusions. The blood urea nitrogen (BUN) was stabilized in several patients, and it appeared that gastrodialysis delayed or prevented the development of the uremic syndrome. In 7 of 14 patients with mean urinary nitrogen excretions of 1.8 g per day, gastrodialysis stabilized a previously rapidly rising blood urea nitrogen for a mean period of 7 days. The stabilization was associated with a maintenance of a uremia-free clinical state. In two of these patients the onset of diuresis led to premature discontinuance of gastrodialysis. In both there was a prompt rise in BUN and the rapid development of uremic symptoms requiring hemodialysis. The following cases selected from the 14 studied provide the best evidence that gastrodialysis may have helped to delay or prevent the symptoms of uremia.

## CASE STUDIES

*Case 1.* A 52 year old man developed acute renal failure following an episode of hypotension during a transurethral resection of the prostate. Gastrodialysis was performed from the seventh to the fourteenth day of oliguria. Simultaneous gastric suction also was carried out. Figure 4 shows the amount of nitrogen removed by gastrodialysis, by gastric suction, and through the urine, and its effect on his serum nonprotein nitrogen (NPN). The sudden rise in the NPN from Day 8 to Day 9 was believed to be due to a 9 L decrease in the size of his extracellular space effected by gastrodialysis plus gastric suction. On Day 11 the patient began to show signs of the uremic syndrome manifested by mental confusion, restlessness and irritability. These signs did not progress. On Day 14 a diuresis began, and he made an uneventful recovery. In this patient, gastrodialysis plus gastric suction removed over 2 g of nitrogen per day and it was associated with a plateau effect on his rising NPN. Also, in addition to removing 15 pounds of extracellular fluid, his serum bicarbonate was maintained at 27 to 30 mEq per L. Potassium excess was never a problem, and sufficient calories were administered to make intravenous feeding unnecessary. It was felt that hemodialysis was averted by gastrodialysis.

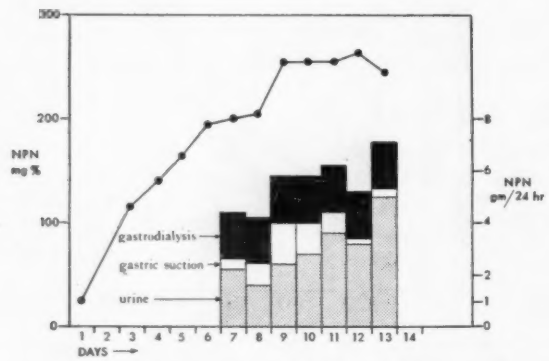



FIG. 4. THE EFFECT OF GASTRODIALYSIS. Case 1, a 52 year old man with acute renal failure complicating transurethral resection of the prostate.

*Case 2.* A 42 year old man developed acute renal failure following exposure to carbon tetrachloride. He was subjected to gastrodialysis continuously from the third to the seventeenth day of oliguria. During this period his urine volume averaged less than 100 ml per day. Potassium removal averaged 21 mEq per day. The patient absorbed 100 to 500 g of glucose per day, the concentration in the bath being 10 per cent. Water removal varied from none to 200 ml per day. In no period was water absorbed by the patient. With the exception of one day when the serum $HCO_3^-$ rose to 41 mEq per L as a result of miscalculation of the amount of HCl in the bath, the serum $HCO_3^-$ was maintained near 25 mEq per L. Nitrogen removal averaged 2.0 g per day. The BUN rose about 10 mg per cent per day and on the tenth day of oliguria was 160 mg per cent. On the following day, because of increasing mental confusion, hemodialysis was carried out with clinical improvement. Gastrodialysis was continued over the next 6 days, and the BUN rose slowly to 144 mg per 100 ml. At this time a urine volume of 500 ml per day was attained, so gastrodialysis was discontinued. However, despite urine volumes averaging 2,700 ml per day, the BUN climbed rapidly over the next 4 days reaching 296 mg per 100 ml. Again, because of symptoms of the uremic syndrome, another hemodialysis was performed. Following this he made a rapid uneventful recovery. The rapid deterioration prior to the second hemodialysis was associated with no event or change in the patient's condition other than discontinuance of gastrodialysis. It seems likely that the second hemodialysis could have been averted had gastrodialysis been continued further into the diuretic period.

*Case 3.* A 3 year old boy developed acute renal failure following an intramuscular injection of bismuth. He was gastrodialyzed for 9 days from the sixth to the fifteenth day of oliguria. During this period his urine volume did not exceed 100 ml per day. His BUN was maintained at 100 mg per 100 ml. On the eighth day of gastrodialysis catabolism increased because of the devel-

opment of bilateral bronchopneumonia. Gastrodialysis was no longer able to handle the increased nitrogen load, and his BUN rose rapidly. A replacement transfusion was carried out on the sixteenth day of oliguria with good results. The patient, however, succumbed to a progressive pneumonia at a time when his urine volume had reached 400 ml per day and recovery from the renal lesion seemed likely. In this patient gastrodialysis seemed effective in controlling his uremia until catabolism increased secondary to the pneumonia.

### COMPLICATIONS

Gastrodialysis was performed in 14 patients studied a total of 65 days. The procedure was generally well tolerated. Complications were infrequent and not of major consequence. One patient developed precordial and epigastric pain considered to be regurgitant esophagitis; this was not a serious problem.

One patient with advanced uremia developed hematemesis following which the cellophane bag was immediately removed and no further bleeding occurred. Postmortem examination performed 2 days later (and also in 6 other patients, each of whom had been subjected to gastrodialysis for many days) revealed no significant pathology in the esophagus or stomach that could be attributed to the procedure.

Two confused patients, while attempting to remove the gastric tube, pulled the cellophane bag off the end of the tube. In neither instance did this result in complication.

One patient developed atelectasis and pneumonitis. It could not be ascertained that gastrodialysis contributed, although this possibility exists.

### DISCUSSION

In the management of patients with acute renal failure, the magnitude of electrolyte transfer by gastrodialysis is adequate to manipulate body water (osmolality), extracellular volume, and acid-base status. In general, the serum sodium is used as a guide to regulation of osmolality and, therefore, water requirements (11). Water losses—sensible, insensible, renal, and to the dialysate—if allowed to go unreplaced, will be accompanied by elevation in the serum sodium concentration. If no alteration of osmolality is desired, it may be necessary to give water to the patient or to decrease the glucose concentration in the dialysate. Since most patients at the outset were hyponatremic, this was rarely necessary.

The extracellular volume was used to determine the need for sodium removal (11), which was undertaken in all patients with edema. Sodium removal was not attempted in patients who did not have systemic edema or pulmonary congestion until it could be ascertained that the extracellular volume was greater than normal. This was done by making use of changes in body weight. The method used requires two assumptions: first, that there is osmotic equality between extra- and intracellular compartments; and second, that in these patients there was continued loss of weight due to catabolism of fat and protein. It was concluded that the extracellular volume was greater than normal if the body weight remained above normal after removal of water restored osmolality to normal. The following is a typical illustration. A man who weighed 70 kg before elective surgery, weighed 79 kg three days after surgery. Systemic edema was not present. At this time his serum sodium concentration was 120 mEq per L. Three days after gastrodialysis was begun his serum sodium concentration had risen to 140 mEq per L and his weight was 72.6 kg. Therefore, his extracellular volume was increased above normal by 2.6 L, plus an amount equal to the weight lost from catabolism. The calculation permits the conclusion that the patient was not saline-depleted and, unless catabolism had been marked, excludes significant saline excess.

In two patients removal of sodium and water by gastrodialysis reduced extracellular volume below normal as judged from decrease in body weight. This was associated with a prompt reversible decrease in urine volume.

If patients were severely acidotic at the outset, 100 mEq per L of sodium bicarbonate was added to the dialysate. Under these circumstances, in 24 hours the patient gained approximately 100 mEq of bicarbonate and lost approximately 100 mEq of hydrogen ions. This was associated with prompt repair of acidosis. After correction, the removal of approximately 100 mEq per 24 hours of hydrogen ions was adequate to prevent recurrence of acidosis.

Potassium removal was adequate to avert hyperkalemia in most patients. However, removal of 25 mEq per day in patients with major catabolic loads associated with infection or injured tissue would prove inadequate to prevent potassium accumulation and hyperkalemia.

The wide variation in transfers, especially hydrogen ion transfers, might suggest that it would

be difficult to arrive at the correct composition of dialysis fluid for a given patient. Actually, this is not true. A dialysis fluid containing 50 mEq per L NaCl and 20 per cent dextrose would result in near zero net transfer of sodium. Water removal would average 200 ml per 24 hours, thus the increase in serum sodium concentration would depend largely upon the magnitude of sensible, insensible, and urinary losses of free water. Hydrogen ion removal would vary with gastric acid production, often being small in the elderly patient. This loss would, in general, be approximately equal to the rate of $H^+$ production secondary to catabolism. Any alkalosis would be easily detected by measurement of the serum bicarbonate and readily corrected by addition of acid to the dialysate. Potassium removal would be small in magnitude and hypokalemia unlikely to result. Even more important is the fact that once the general range of transfers was established for any given patient, the range of variation was small. Sudden, unexpected transfers were not encountered.

It is our impression that gastrodialysis was beneficial in delaying the onset of the uremic syndrome. In three patients with urine volumes of less than 300 ml per 24 hours, gastrodialysis was continued for five, seven and ten days, respectively. None required hemodialysis. It seems likely that the clinical course was better than could be expected with conservative management alone.

In one patient, severely injured and with a large catabolic load, gastrodialysis was ineffectual in both potassium removal and in prevention of uremia.

Recently we have undertaken prophylactic hemodialysis in the management of patients with acute renal failure (12, 13). The goal is to prevent any manifestation of the uremic syndrome and thus provide the patient with optimal opportunity to recover from his basic illness. Preliminary experience with prophylactic hemodialysis has been encouraging. If this approach proves sound, gastrodialysis would be effectual only in those patients who had no increase in catabolism secondary to the basic disease process, i.e., infection or trauma. Unfortunately, this group is a small minority of all cases of acute renal failure.

## SUMMARY AND CONCLUSIONS

A technique of gastrodialysis has been described that is safe, practical, and without serious complications.

Although probably the least efficient of present-day methods of dialysis, gastrodialysis has proven useful in the treatment of certain aspects of acute renal failure. Gastrodialysis is satisfactory as a means of regulation of osmolality, extracellular volume, and acid-base status. Dextrose can be given by the alimentary route and without administration of water.

The removal of nitrogen and potassium does not compare favorably with extracorporeal dialysis. However, in patients without a large catabolic load, gastrodialysis appears adequate to prevent hyperkalemia and to avoid or decrease the number of hemodialyses required.

Gastrodialysis, to be effective, must be begun early and continued throughout the course of acute renal failure. Hemodialysis should also be available in case the patient cannot be maintained by means of gastrodialysis.

## ACKNOWLEDGMENTS

The authors wish to acknowledge the technical assistance of Patricia L. Hoover, Jessie Blackie and Kiku Kusabe.

## REFERENCES

1. Schloerb, P. R. Gastrodialysis with a semi-permeable membrane bag. Clin. Res. Proc. 1957, **5**, 196.
2. Schloerb, P. R. Enterodialysis: An approach to the problem of renal substitution. Surg. Forum 1956, **7**, 58.
3. Miller, G. L., and Miller, E. E. Determination of nitrogen in biological materials (improved Kjeldahl-Nessler method). Analyt. Chem. 1948, **20**, 481.
4. Vanselow, A. P. Preparation of Nessler's reagent. J. industr. engin. Chem., Anal. Ed. 1940, **12**, 516.
5. Scribner, B. H. Bedside determination of chloride: A method for plasma, urine, and other fluids and its application to fluid balance problems. Proc. Mayo Clin. 1950, **25**, 209.
6. Scribner, B. H., and Caillouette, J. C. Improved method for the bedside determination of bicarbonate in serum. J. Amer. med. Ass. 1954, **155**, 644.
7. Hawk, P. B., and Bergeim, O. Practical Physiological Chemistry, 11th ed. Philadelphia, The Blakiston Company, 1937, p. 317.

8. Official Methods of Analysis of the Association of Official Agricultural Chemists, 8th ed., W. Horwitz, Ed. Washington, D. C., 1955, p. 532, item 29.5.
9. Benedict, S. R. Analysis of whole blood; the determination of sugar and of saccharoids (nonfermentable copper reducing substances). J. biol. Chem. 1931, **92**, 141.
10. Burnell, J. M., Villamil, M. F., Uyeno, B. T., and Scribner, B. H. The effect in humans of extracellular pH change on the relationship between serum potassium concentration and intracellular potassium. J. clin. Invest. 1956, **35**, 935.
11. Burnell, J. M., Paton, R., and Scribner, B. H. The problem of sodium and water needs of patients. J. chron. Dis. In press.
12. Scribner, B. H. Role of the artificial kidney in management of acute renal failure. Northw. Med. (Seattle) 1959, **58**, 555.
13. Teschan, P. E., O'Brien, T. F., and Baxter, C. R. Prophylactic daily hemodialysis in the treatment of acute renal failure. Clin. Res. 1959, **7**, 280.

# CALCIUM AND PHOSPHORUS HOMEOSTASIS IN THE PARATHYROIDECTOMIZED DOG; EVALUATION BY MEANS OF ETHYLENEDIAMINE TETRAACETATE AND CALCIUM TOLERANCE TESTS *

By PETER H. SANDERSON,† FOSTER MARSHALL, II ‡ and RICHARD E. WILSON §

*(From the Department of Surgery of the Harvard Medical School at the Peter Bent Brigham Hospital, Boston, Mass.)*

(Submitted for publication August 28, 1959; accepted December 10, 1959)

In an experiment designed to determine whether or not homografts of parathyroid tissue would survive and function in millipore diffusion chambers, Wilson, Zollinger, Mahan and Brooks (1) subjected female mongrel dogs to parathyroidectomy either in two stages or as a single procedure. Twelve animals with transplants survived the acute postoperative period with its attendant severe hypocalcemia, but all required intensive parenteral calcium support for periods ranging from 19 to 78 days. This supportive therapy could eventually be stopped, and 11 of these dogs were perfectly well in all respects for two to five months prior to removal of their transplants. The range of the serum calcium level was 9 to 11 mg per 100 ml in six of them, and 7 to 8 mg per 100 ml in the other five. However, after all of the millipore chambers were removed, there was little or no alteration in their clinical status or their serum calcium levels. The dogs were followed for two and one-half to four months after the chambers were excised, and the only shift in the level of serum calcium that occurred was in one dog, which, with a serum calcium initially in the 7 to 8 mg per 100 ml range, was later able to maintain a consistent value of 9.0 mg per 100 ml.

* This work was supported in part by Grant A-1425 from the United States Public Health Service and by a grant from the Atomic Energy Commission (AT (30-1)-2265) to the Peter Bent Brigham Hospital.

† Research Fellow in Surgery, Harvard Medical School; Assistant in Surgery, Peter Bent Brigham Hospital; George Gorham Peters Fellow from St. Mary's Hospital, London, England.

‡ Research Fellow in Surgery, Harvard Medical School; Assistant in Surgery, Peter Bent Brigham Hospital; Harvey Cushing Fellow.

§ Research Fellow in Surgery, Harvard Medical School; Assistant in Surgery, Peter Bent Brigham Hospital; U. S. Public Health Service Research Fellow of the National Cancer Institute.

It might be argued that these animals were being kept alive by accessory parathyroid tissue. However, the usual site of any such tissue (the thyroid gland) was always removed. In addition, whenever one parathyroid gland was deliberately left in place, the serum calcium level remained normal for as long as 140 days; yet, in every dog in which parathyroidectomy was felt to be complete, a precipitous fall in the level of serum calcium occurred within 48 hours, and animals that were not given calcium injections at this stage died within eight days.

A further possibility is that small amounts of parathyroid tissue, insufficient at the time of operation to sustain life, were left and subsequently underwent hypertrophy while the animal was being kept alive by calcium injections. In view of the high proportion (55 per cent) of dogs in which this explanation would need to be invoked we regard it as somewhat unlikely. We believe, therefore, that the surviving dogs became adapted to existence without any parathyroid tissue whatever. While all had normal appearance and did not exhibit latent tetany, six of the eleven dogs were able to maintain serum calcium values in the normal range, and the other five stabilized their serum calcium at a lower level.

These findings pose two major problems. First, how is parathyroid function assessed in the dog, and how do these adapted animals differ from the normal in their regulation of calcium and phosphorus metabolism? Secondly, what are the mechanisms whereby aparathyroid dogs can exert control over the metabolism of calcium and phosphorus? It has been the purpose of this study to concentrate primarily on the first of these questions.

## MATERIALS AND METHODS

Ten female mongrel dogs, with weights ranging from 10 to 20 kg, had previously undergone total thyro-para-

thyroidectomy and had reached the adapted state just described (1). These are referred to below as the experimental or parathyroidectomized dogs. Eight normal female mongrel dogs, with weights between 11.8 and 19 kg, served as controls. Perineotomy was performed on all animals, both experimental and control, to facilitate urethral catheterization. All the dogs received a normal kennel diet, which consisted of Ken-L-Bisket (Quaker Oats), kibbled medium, 2 to 4 cups per day, with water *ad libitum*. Their estimated daily calcium intake was of the order of 2.5 g.

*Measurement of maximum tubular reabsorption of phosphorus* ($T_mP$). Fifty ml of water per kg of body weight was administered by stomach tube. One hour later, an intravenous infusion was set up and a priming injection, consisting of 4 ml of 4 per cent creatinine and 3.5 ml of 0.5 M sodium phosphate per kg of body weight, was given. This was followed by a sustaining injection of 4 ml of 4 per cent creatinine and 4 ml of 0.5 M sodium phosphate per kg, made up to 500 ml with 0.9 per cent sodium chloride solution and given at the rate of 6 ml per minute. The sodium phosphate consisted of $Na_2HPO_4 \cdot 12\ H_2O$ and $NaH_2PO_4 \cdot H_2O$ in the proportion of 10.9 : 1 Twenty minutes was allowed for the establishment of a steady state, and then three or four 15 to 20 minute urine collections were made via an indwelling Foley catheter. Blood samples were drawn at the midpoint of each period.

*Phosphorus: creatinine clearance ratio* ($C_P/C_{Cr}$) *after intravenous calcium infusion.* A Foley urethral catheter was passed, and the bladder was emptied and washed out with two 50 ml volumes of distilled water; the catheter was then clamped and the urine and bladder washings discarded. One hour later, the bladder was again emptied and washed out; the combined urine and washings constituted the pre-infusion specimen. An intravenous infusion containing 15 mg of calcium per kg of body weight (as calcium gluconate [1]), made up to a total volume of 120 ml with 0.9 per cent sodium chloride solution, was then started. The rate of flow was adjusted so that the infusion was completed in 1 hour. Urine samples were collected, with washings, at the end of the infusion, 1 hour later, and thereafter at intervals of 2 hours for 6 more hours. Blood samples were collected at the midpoint of the pre-infusion period, at the end of the infusion, and at intervals of 2 hours thereafter for 6 more hours. In most of these experiments a further blood sample was collected 24 hours after the end of the infusion.

*Infusion of ethylenediamine hydrogen trisodium acetate (EDTA trisodium salt, Trisodium Versenate).* The procedure was similar to that for calcium infusion, except that urine samples were collected for 1 hour before the infusion, and at intervals of 2 hours after the start of the infusion for a total of 8 hours. During the one hour infusion period, 50 mg per kg of EDTA trisodium salt, or the equivalent weight of the disodium salt,[2] dissolved in 120 ml of 0.9 per cent sodium chloride solution, was injected. Blood samples were drawn at the midpoint of the pre-infusion period, at the end of the infusion, and 1, 2, 4, 6 and 24 hours afterwards.

*Chemical methods.* Serum calcium was determined by compleximetric titration, with ammonium purpurate as the indicator, as devised by Munson and associates (2). Phosphorus in the serum and urine was measured by the method of Gomori (3). Creatinine was determined in two ways. In the $T_mP$ experiments, with exogenous creatinine loading and high serum and urine creatinine levels, the method of Folin and Wu (4) was used, modified as follows: serum proteins were removed by "acid tungstate" precipitation, with 1 : 4 dilution, as recommended by Owen, Iggo, Scandrett and Stewart (5) and the color developed by taking 0.2 ml filtrate, 3.8 ml water, and 2 ml of alkaline picrate. Urines were diluted appropriately and treated with alkaline picrate in the same manner. In the calcium and EDTA infusion experiments, where the serum creatinine level was not artificially elevated, the adsorption-elution procedure with Lloyd's reagent, described by Owen and co-workers (5), was followed exactly, except that in most of the experiments serum proteins were removed by trichloroacetic acid at a 1 : 4 dilution, with a final concentration of 10 per cent trichloroacetic acid.

## RESULTS

*Serum calcium.* The mean serum calcium in the normal dogs, calculated from two determinations in each of six animals, was 10.8 mg per 100

TABLE I

*Tubular maximal reabsorption of phosphorus* ($T_mP$)

| Dog | Range of serum inorganic phosphorus | Periods | $T_mP$* |
|---|---|---|---|
| *no.* | *mg/100 ml* | *no.* | |
| Parathyroidectomized | | | |
| 2 | 24.0–24.3 | 3 | 85 |
| 4 | 21.8–22.3 | 3 | 148 |
| 5 | 21.8–23.6 | 4 | 167 |
| 6 | 26.2–26.6 | 3 | 154 |
| 12 | 24.0–25.2 | 4 | 92 |
| 13 | 18.8–21.4 | 4 | 79 |
| | | | Mean 121 |
| Normal | | | |
| 102 | 18.8–20.3 | 3 | 111 |
| 104 | 15.7–17.1 | 4 | 97 |
| 106 | 17.4–18.2 | 3 | 169 |
| | | | Mean 125 |

* Expressed as micromoles of phosphorus per minute, corrected to a glomerular filtration rate of 100 ml per minute.

[1] Kindly supplied by Parke, Davis & Company, Detroit, Mich.

[2] A generous supply of the latter substance was provided by Riker Laboratories, Inc., Los Angeles, Calif.

TABLE II

*Clearance data following infusion of calcium (15 mg/kg) during one hour*

| Dog | Periods 1 1 Hr before infusion | 2 1 Hr during infusion | 3 0–1 Hr after infusion | 4 1–3 Hrs after infusion | 5 3–5 Hrs after infusion | 6 5–7 Hrs after infusion |
|---|---|---|---|---|---|---|
| no. | | | Creatinine clearance values | | | |
| Parathyroidectomized | | | | | | |
| 1 | 45.5 | 40.8 | 40.2 | 45.0 | 42.6 | 54.0 |
| 4 | 33.8 | 31.8 | 25.0 | * | 27.3 | 35.3 |
| 5 | 70.2 | 74.5 | 65.2 | 67.0 | 63.3 | 72.5 |
| 12 | 42.3 | 51.0 | 49.5 | 45.4 | 44.1 | 36.0 |
| 15 | 33.3 | 39.6 | 39.4 | 38.3 | 34.2 | 40.8 |
| 21 | 50.7 | 40.0 | 43.3 | 39.0 | 35.4 | 47.1 |
| 23 | * | * | 23.4 | 22.0 | 24.3 | 30.2 |
| Normal | | | | | | |
| 102 | 22.1 | 36.8 | 36.0 | 32.6 | 32.2 | 27.0 |
| 104 | 33.1 | 35.6 | 38.4 | 33.3 | 31.3 | 33.5 |
| 105 | 44.0 | 42.7 | 47.3 | 47.4 | 49.5 | 59.0 |
| 106 | 36.7 | 43.5 | 41.4 | * | * | * |
| 107 | 46.1 | 47.3 | 51.2 | 55.3 | 45.6 | 56.8 |
| 109 | 52.5 | 54.4 | 43.6 | 50.1 | 43.0 | 38.4 |
| | | | Phosphorus: Creatinine clearance ratios† | | | |
| Parathyroidectomized | | | | | | |
| 1 | 0.293 | | 0.280 | 0.171 | 0.081 | 0.072 |
| 4 | 0.282 | 0.172 | 0.103 | 0.137 | 0.148 | 0.146 |
| 5 | 0.160 | 0.125 | 0.111 | 0.100 | 0.132 | 0.096 |
| 12 | 0.166 | 0.087 | 0.192 | 0.154 | 0.146 | 0.129 |
| 15 | 0.210 | 0.090 | 0.051 | 0.0435 | 0.057 | 0.050 |
| 21 | 0.113 | 0.072 | 0.058 | 0.076 | 0.0795 | 0.0644 |
| 23 | 0.183 | 0.027 | 0.096 | 0.138 | 0.109 | 0.090 |
| Normal | | | | | | |
| 102 | 0.164 | 0.060 | 0.019 | 0.0053 | 0.0037 | 0.0022 |
| 104 | 0.228 | 0.116 | 0.0428 | 0.0474 | 0.250 | 0.195 |
| 105 | 0.241 | 0.096 | 0.055 | 0.142 | 0.08 | 0.046 |
| 106 | 0.0502 | 0.0053 | 0.00224 | 0.00155 | 0.00296 | 0.0509 |
| 107 | 0.0317 | 0.0029 | 0.0344 | 0.212 | 0.125 | 0.515 |
| 109 | 0.0416 | 0.044 | 0.079 | 0.133 | 0.182 | 0.115 |

* Incomplete collection of urine.

† Calculated as $\frac{P_u \times Cr_p}{Cr_u \times P_p}$. This ratio may be converted to percentage of tubular reabsorption of phosphorus by the following relation: $\%$ T.R.P. $= 100 - (C_P/C_{Cr} \times 100)$.

ml (SD ± 0.55). Of the parathyroidectomized dogs, six usually had serum calcium levels ranging between 9.0 and 11.0 mg per 100 ml, although slightly lower values were sometimes obtained. The levels in the remaining four (nos. 4, 5, 21 and 23) were always lower and ranged between 5.8 and 8.3 mg per 100 ml.

*Serum phosphorus.* The mean serum inorganic phosphorus level in normal dogs (based upon 11 determinations in 6 animals) was 4.6 mg per 100 ml (SD ± 0.8). In the parathyroidectomized dogs (10 animals, 19 determinations) the mean level was 5.6 mg per 100 ml (SD ± 1.3). The difference between these means was statistically significant ($p < 0.05$). However, in the six parathyroidectomized dogs with more normal serum calcium values, the mean phosphorus level was 4.7 mg per 100 ml, whereas in the four with lower calcium levels, the mean phosphorus value was 6.8 mg per 100 ml ($p < 0.001$).

*Tubular maximal reabsorption of phosphorus ($T_mP$).* The results are shown in Table I. It will be seen that in all the experiments the serum inorganic phosphorus levels were over 15 mg per 100 ml and in the majority were near 20 mg per 100 ml. These levels were found satisfactory by Hogben and Bollman (6) for the measurement of phosphorus Tm in the dog. There was essentially

no difference in the findings for the normal and the parathyroidectomized dogs.

*Phosphorus: creatinine clearance ratio ($C_P/C_{Cr}$) after calcium infusion.* These results are shown in Table II. It will be seen that in all the dogs studied, except 109, the ratio fell during the experiment. However, the results do not provide a clear-cut distinction between the normal and the parathyroidectomized dogs. In Dogs 102, 104 and 106, the ratio decreased more than in any of the others, falling to 1.3, 3.2 and 6.4 per cent, respectively, of the level before the calcium infusion. This pronounced decrease is in harmony with the results obtained in normal man, and it probably indicates the presence of functioning parathyroid tissue. Its absence, under the conditions of our experiments, does not indicate the contrary.

*Serum calcium levels after calcium infusion.* The results are shown in Table III and Figure 1. The usual statistical techniques have been applied to these data, and the results are shown in the values for p at the foot of the table. These indicate highly significant differences between the normal and the parathyroidectomized dogs for all periods of the experiment. Parathyroidectomized dogs with low initial serum calcium levels demonstrated the same response as the other parathyroidectomized dogs when the results were expressed as percentages of the initial value.

*Serum phosphorus levels after calcium infusion.* The results are shown in Table IV. It is clear that a conspicuous rise in serum phosphorus occurred in both groups, reaching a peak one hour after the end of the infusion, and then declining. No difference in this respect could be discerned between the two groups.

*Serum calcium levels (non-EDTA-bound) after infusion of EDTA sodium salt.* The results are shown in Table V and Figure 1. The

TABLE III

*Serum calcium levels following infusion of calcium (15 mg/kg) during one hour*

| Dog | Initial value | End of infusion | | 2 Hrs after infusion | | 4 Hrs after infusion | | 6 Hrs after infusion | | 24 Hrs after infusion | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % Init. value | | % Init. value | | % Init. value | | % Init. value | | % Init. value |
| *no.* | *mg/100 ml* | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | |
| Parathyroidectomized | | | | | | | | | | | |
| 1 | 9.6 | 12.4 | 129 | 10.9 | 114 | 10.3 | 108 | 9.4 | 98 | 9.4 | 98 |
| 2 | 9.4 | 15.3 | 163 | 13.6 | 145 | 12.6 | 134 | 12.0 | 128 | 10.9 | 116 |
| 4 | 7.1 | 11.5 | 162 | 11.1 | 156 | 10.5 | 148 | 9.1 | 128 | | |
| 5 | 8.3 | 11.9 | 144 | 11.2 | 135 | 11.1 | 134 | 10.5 | 127 | 9.6 | 116 |
| 6 | 8.8 | 13.2 | 150 | 11.9 | 135 | 11.2 | 127 | 10.8 | 123 | 9.7 | 110 |
| 12 | 10.3 | 16.7 | 162 | 15.1 | 147 | 13.1 | 127 | | | 10.8 | 105 |
| 13 | 10.8 | 14.5 | 134 | 13.3 | 123 | 12.7 | 118 | 11.9 | 110 | | |
| 15 | 8.5 | 13.3 | 157 | 12.3 | 145 | 11.5 | 135 | 11.4 | 134 | 10.3 | 121 |
| 21 | 6.7 | 10.9 | 163 | 10.3 | 154 | 9.9 | 148 | 10.0 | 149 | 8.2 | 122 |
| 23 | 7.2 | 14.1 | 196 | 11.8 | 164 | 9.3 | 129 | 8.4 | 117 | 7.8 | 108 |
| Mean | | | 156.0 ±18.8 | | 141.8 ±15.4 | | 130.6 ±12.2 | | 123.8 ±14.4 | | 112.0 ± 8.1 |
| Normal | | | | | | | | | | | |
| 102 | 11.4 | 15.1 | 133 | | | 11.5 | 101 | 10.0 | 88 | 10.6 | 93 |
| 104 | 10.7 | 12.1 | 113 | 10.9 | 102 | 10.4 | 97 | 10.7 | 100 | 10.8 | 101 |
| 105 | 10.5 | 14.8 | 141 | | | 12.4 | 118 | | | 10.8 | 103 |
| 106 | 10.1 | 12.7 | 126 | 11.0 | 109 | 10.1 | 100 | 10.2 | 101 | 9.6 | 95 |
| 107 | 10.9 | 12.4 | 114 | 11.6 | 105 | 11.0 | 101 | 10.7 | 98 | 10.9 | 100 |
| 109 | 11.0 | 14.3 | 130 | 11.8 | 107 | 10.6 | 96 | 10.9 | 99 | 10.9 | 99 |
| Mean | | | 126.2 ±11.5 | | 105.8 ± 2.9 | | 102.2 ± 6.6 | | 97.2 ± 5.3 | | 98.5 ± 5.4 |
| p Values for differences between the means | | | | | | | | | | | |
| Upper limit | | | 0.01 | | 0.005 | | 0.001 | | 0.005 | | 0.01 |
| Lower limit | | | 0.005 | | 0.001 | | | | 0.001 | | 0.005 |

TABLE IV

*Serum phosphorus levels following infusion of calcium (15 mg/kg) during one hour*

| Dog | Initial value | End of infusion | | 2 Hrs after infusion | | 4 Hrs after infusion | | 6 Hrs after infusion | | 24 Hrs after infusion | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % Init. value | | % Init. value | | % Init. value | | % Init. value | | % Init. value |
| no. | *mg/100 ml* | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | |
| Parathyroidectomized | | | | | | | | | | | |
| 1 | 4.9 | 4.8 | 98 | 4.3 | 88 | 4.2 | 86 | 3.3 | 67 | | |
| 2 | 5.1 | 5.1 | 100 | 6.5 | 128 | 6.8 | 134 | 6.4 | 126 | 6.3 | 124 |
| 4 | 7.6 | 7.2 | 95 | 10.0 | 132 | 9.1 | 120 | 9.0 | 118 | | |
| 5 | 6.0 | 7.3 | 122 | 8.4 | 140 | 7.5 | 125 | 8.2 | 137 | 7.4 | 123 |
| 6 | 6.3 | 5.6 | 106 | 6.9 | 130 | 6.1 | 115 | 5.9 | 111 | 5.3 | 100 |
| 12 | 4.5 | 6.0 | 133 | 6.2 | 138 | 5.8 | 129 | | | 6.2 | 138 |
| 13 | 3.6 | 4.3 | 119 | 5.9 | 164 | 6.2 | 172 | 6.4 | 178 | | |
| 15 | 4.8 | 5.1 | 106 | 6.5 | 135 | 6.9 | 144 | 6.7 | 139 | 5.7 | 119 |
| 21 | 6.1 | 6.5 | 107 | 7.7 | 126 | 8.2 | 134 | 6.5 | 107 | 6.2 | 102 |
| 23 | 7.5 | 8.8 | 117 | 9.4 | 125 | 9.9 | 132 | 8.5 | 113 | 7.3 | 98 |
| Mean | | | 110.3 ±12.5 | | 130.6 ±19.2 | | 129.1 ±22.3 | | 121.8 ±30.3 | | 114.9 ±15.2 |
| Normal | | | | | | | | | | | |
| 102 | 4.8 | 4.6 | 96 | 5.5 | 115 | 5.2 | 108 | 5.5 | 115 | 4.8 | 100 |
| 104 | 6.2 | 6.4 | 103 | 7.0 | 113 | 6.8 | 110 | 6.1 | 99 | 6.2 | 100 |
| 105 | 5.3 | 5.0 | 94 | 6.8 | 128 | 5.8 | 109 | 6.3 | 119 | 4.7 | 89 |
| 106 | 4.9 | 5.4 | 110 | 4.8 | 98 | 4.8 | 98 | 4.6 | 94 | 5.2 | 106 |
| 107 | 3.9 | 6.3 | 162 | 6.9 | 177 | 6.7 | 172 | 6.2 | 159 | | |
| 109 | 4.1 | 5.3 | 129 | 6.2 | 151 | 5.6 | 137 | 4.5 | 110 | | |
| Mean | | | 115.7 ±25.7 | | 130.3 ±28.2 | | 122.3 ±25.2 | | 116.0 ±21.9 | | 98.8 ± 7.2 |

values of p for the differences between the two groups have been determined as in Table III, and it can be seen that these differences are highly significant, except for the levels at the end of the infusion. The slow restoration of the original calcium level in the parathyroidectomized dogs is obvious from Figure 1. As with the calcium infusions, the responses of the parathyroidectomized dogs with a low initial serum calcium level could not be separated from those of the other parathyroidectomized dogs, when the results are expressed as percentages of the initial values. The serum calcium level was not followed in every dog to the point of restoration of the initial value. In one animal, however, the process was studied in detail, and the results (Table VI) give a good indication of the sluggishness of this response.

*Serum inorganic phosphorus levels after infusion of EDTA sodium salt.* No consistent changes were seen in either the normal or the parathyroidectomized dogs.

*Effect of thyroid replacement.* It might be argued that the altered response to calcium and to EDTA infusions in the operated animals was due

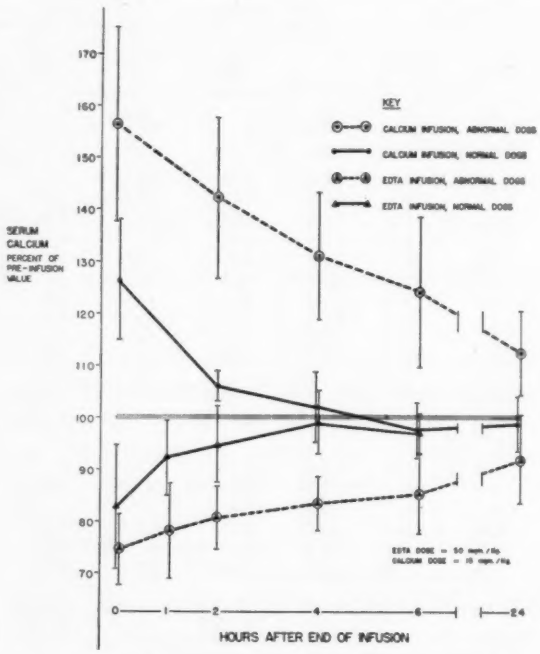



FIG. 1. MEAN SERUM CALCIUM LEVELS, EXPRESSED AS PERCENTAGES OF PRE-INFUSION VALUES, FOLLOWING CALCIUM AND EDTA INFUSIONS IN NORMAL AND IN ADAPTED PARATHYROIDECTOMIZED DOGS. The vertical lines represent one standard deviation above and below the means.

TABLE V

*Serum calcium levels (non-EDTA-bound) following infusion of EDTA trisodium salt (50 mg/kg) during one hour*

| Dog | Initial value | End of infusion | % Init. value | 1 Hr after infusion | % Init. value | 2 Hrs after infusion | % Init. value | 4 Hrs after infusion | % Init. value | 6 Hrs after infusion | % Init. value | 24 Hrs after infusion | % Init. value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *no.* | *mg/100 ml* | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | | *mg/100 ml* | |
| Parathyroidectomized | | | | | | | | | | | | | |
| 1 | 10.9 | 8.7 | 80 | 9.0 | 83 | 8.9 | 82 | 8.6 | 79 | 8.4 | 77 | 8.9 | 82 |
| 2 | 10.1 | 8.3 | 82 | 7.8 | 77 | 7.3 | 79 | 7.9 | 78 | 7.7 | 76 | 7.7 | 76 |
| 4 | 6.9 | 4.8 | 70 | 4.0 | 58 | 5.0 | 72 | 5.8 | 84 | | | 6.5 | 94 |
| 5 | 5.8 | 4.3 | 74 | 4.7 | 81 | 4.8 | 83 | 4.9 | 84 | 5.1 | 88 | 5.5 | 95 |
| 6 | 8.2 | 5.6 | 68 | 6.5 | 79 | 6.6 | 81 | 7.0 | 85 | 7.2 | 88 | 8.0 | 98 |
| 12 | 9.6 | 7.0 | 73 | 7.7 | 88 | 7.8 | 81 | 7.8 | 81 | 8.4 | 88 | 8.7 | 91 |
| 13 | 9.9 | 8.3 | 84 | 9.1 | 92 | 9.1 | 92 | 9.2 | 93 | 9.7 | 98 | 9.9 | 100 |
| 15 | 9.0 | 6.9 | 77 | 7.3 | 81 | 7.5 | 83 | 7.4 | 82 | 7.7 | 86 | | |
| 21 | 8.2 | 6.3 | 77 | 6.7 | 82 | 6.8 | 83 | 7.3 | 89 | 7.4 | 90 | 8.4 | 102 |
| 23 | 6.3 | 4.1 | 65 | 4.7 | 75 | 5.3 | 84 | 5.2 | 83 | 5.2 | 83 | 6.0 | 95 |
| Mean | | | 75.0 ± 6.8 | | 78.8 ±9.1 | | 81.3 ±6.0 | | 83.8 ±5.1 | | 86.0 ±7.5 | | 92.6 ±8.5 |
| Normal | | | | | | | | | | | | | |
| 102 | 10.2 | 9.2 | 90 | 10.1 | 99 | 10.5 | 103 | 11.0 | 108 | 9.6 | 94 | | |
| 104 | 11.3 | 9.8 | 87 | 10.6 | 94 | 11.1 | 98 | 10.7 | 95 | | | 10.7 | 95 |
| 105 | 10.5 | 7.8 | 74 | 8.4 | 80 | 9.1 | 86 | 9.7 | 92 | 10.1 | 96 | 10.2 | 97 |
| 106 | 10.7 | 10.1 | 94 | 10.6 | 99 | 10.5 | 98 | 10.3 | 96 | 10.0 | 94 | | |
| 107 | 11.6 | 10.4 | 90 | 10.2 | 88 | 10.6 | 91 | 11.7 | 101 | 11.6 | 100 | | |
| 109 | 10.0 | 6.3 | 63 | 9.4 | 94 | 9.6 | 96 | 10.3 | 103 | 10.3 | 103 | | |
| Mean | | | 83.0 ±11.9 | | 92.4 ±7.3 | | 95.3 ±7.3 | | 99.2 ±6.1 | | 97.4 ±3.9 | | |
| p Values for differences between the means | | | | | | | | | | | | | |
| Upper limit | | | 0.2 | | 0.01 | | 0.005 | | 0.001 | | 0.01 | | |
| Lower limit | | | 0.1 | | 0.005 | | 0.001 | | | | 0.005 | | |

to lack of thyroid rather than parathyroid hormone, since, in every case, the thyroid gland was removed as completely as possible. In some of the parathyroidectomized dogs, the studies were carried out while the animals were receiving thyroid extract orally (30 mg three times a week, from the time of thyroidectomy). The remaining dogs were studied at periods up to four months after thyroid replacement had been discontinued. No difference could be found between the responses of dogs with and without thyroid replacement.

TABLE VI

*EDTA infusion in Dog. no. 2*

| Time | | Non-EDTA-bound serum calcium |
|---|---|---|
| | *Days* | *mg/100 ml* |
| Before infusion | | 10.1 |
| At end of infusion | | 8.3 |
| After infusion: | 1 | 7.7 |
| | 2 | 8.6 |
| | 3 | 8.7 |
| | 4 | 8.9 |
| | 5 | 9.2 |
| | 6 | 9.6 |
| | 7 | 9.8 |
| | 8 | 10.2 |

In order to explore this point more thoroughly, two dogs, which had previously been tested without thyroid replacement were given triiodothyronine orally and then tested. One dog received 25 μg daily for 5 days, then 50 μg daily for 16 days; the other was given 50 μg daily for 16 days. The responses of these animals to calcium and EDTA infusions were unchanged by triiodothyronine in this dosage. To exclude the possibility that the drug was not being absorbed properly, the same two dogs were treated with intramuscular injections of 120 μg of triiodothyronine daily for 7 days, and the infusions were then repeated. The responses were again essentially unchanged.

## DISCUSSION

It is clear that, from the standpoint of separating normal from parathyroidectomized dogs, some criteria were more satisfactory than others. The level of serum calcium, for example, was quite unreliable; six out of the ten parathyroidectomized dogs consistently had normal values after an initial period of critical and life-endangering hypocalcemia. It is of interest that the same six dogs also had normal levels of serum inorganic phosphorus.

The tubular maximal reabsorption of phosphorus was also an unsatisfactory criterion. However, this finding was not entirely unsuspected, since attempts to detect hypoparathyroidism in humans by means of this test, in the experience of most workers (7), have been unsuccessful. Hogben and Bollman (6) demonstrated a raised $T_mP$ in parathyroidectomized dogs, but these measurements were made some 14 days after operation, and it is therefore probable that their animals had not reached the adapted state.

The phosphorus: creatinine clearance ratio after calcium infusion has been used for some years to evaluate parathyroid function in man, and is a valuable clinical test (8, 9). The response seen in normal subjects is a rise in serum inorganic phosphorus and a fall in $C_P/C_{Cr}$, while in hypoparathyroid subjects, although the rise in serum phosphate occurs, the $C_P/C_{Cr}$ remains unchanged or may even rise.

Our observations in dogs are not consistent with the findings in man. It is possible that a species difference is responsible, but it should also be noted that the conditions of our test were somewhat different from those originally proposed by Howard, Hopkins and Connor (8). While the dose of calcium (15 mg per kg) was the same, we have given the injection over a period of one hour rather than four hours, and we have confined our measurements to the six hours following the infusion. Some preliminary studies of $C_P/C_{Cr}$, 24 hours after the infusion, showed that no additional information was obtained. It is possible that the longer stimulus of the four hour infusion would have produced a sharp reduction in $C_P/C_{Cr}$ in all of our normal dogs, instead of in only half of them. The rise in serum phosphorus in both the normal and the parathyroidectomized dogs after calcium infusion is comparable with the changes seen in normal humans and in patients with hypoparathyroidism by Nordin and Fraser (9) and supports the view that the parathyroid glands are not responsible for this phenomenon.

The changes in serum calcium levels produced by infusions of calcium and of EDTA were more satisfactory as a means of discriminating between normal and parathyroidectomized dogs. Many investigators have given infusions of calcium to normal humans and to patients with hypoparathyroidism (8–10); but attention seems to have been focused mainly on the changes in urinary phosphate excretion, and little account has been taken of the serum calcium levels. This consideration, and the fact that our calcium infusions were given in one quarter of the time customary for studies in man, make it difficult to relate our findings to human parathyroid physiology. In the dog, however, it is clear that while the parathyroid glands may not be necessary for life or even for the maintenance of a normal serum calcium level, in their absence the animal is unable to deal so effectively with an acute intravenous load of calcium.

In interpreting the changes in serum calcium following EDTA infusion, it must be recognized that the method used to determine serum calcium under these circumstances, measures not the total calcium but only that fraction of it that is not already bound by EDTA. Had the EDTA in the serum been destroyed by preliminary ashing before the estimates were made, the figures obtained for serum calcium would have been substantially higher. However, EDTA infused *in vivo* probably binds the ionized fraction of serum calcium more effectively than any other, and the fall in non-EDTA-bound calcium in these dogs was almost certainly accompanied by a fall in the ionized fraction. There is some evidence (11, 12) that variations in this fraction may be responsible for regulating the output of parathyroid hormone. Our results show that the normal animal rapidly restores the non-EDTA-bound fraction (and probably the ionized fraction) to its original level, while the parathyroidectomized animal does so only slowly and imperfectly.

Somewhat similar findings were recorded by

Patt and Luckhardt (11) and Stewart and Bowen (12) who infused sodium oxalate solutions into normal and parathyroidectomized dogs, and showed that in the latter group restoration of the serum calcium level was slow and incomplete. The thyroid and parathyroid glands of these dogs had been removed shortly before the infusions; in the study of Patt and Luckhardt, just prior to the infusions, and in that of Stewart and Bowen, one to five days previously. From our results it is evident that this disability in calcium regulation persists indefinitely. On the other hand, Copp (13) performed similar experiments on acutely parathyroidectomized dogs, using larger doses of EDTA (equivalent to 86 mg per kg of the trisodium salt). He was unable to discern any obvious alteration in the response to EDTA infused 20 hours after parathyroidectomy.

If the results from the infusions of calcium and EDTA are considered together, it becomes clear that the homeostatic mechanisms responsible for maintaining the level of serum calcium constant are vulnerable to acute challenge in the parathyroidectomized dog as compared with the normal. This is the case whether the serum calcium is raised or lowered from its initial level. At the same time, the parathyroidectomized dogs do possess a mechanism capable of restoring their level of serum calcium toward the original value.

Our study does not permit any conclusion as to the mechanism of this homeostasis in the intact dog. Only two basic processes appear possible in such a rapid adjustment. There is either a renal mechanism, whereby the rate of urinary calcium excretion is increased or decreased according to the needs of the situation, or an alteration of the equilibrium between the circulating calcium and the calcium in the skeleton. Previous work (14–16) indicates that parathyroid hormone may take effect through both mechanisms. In the parathyroidectomized dogs, as has been noted above, there is good reason to believe that no parthyroid tissue remained; yet the animals were still able to regulate their serum calcium levels, although with less than normal efficiency. This implies one of two possibilities. On the one hand, these dogs may have been obtaining parathyroid hormone, or some substance with the same biological action, from other tissues, as has been postulated in some human patients with neoplasms (17, 18). On the other hand, the removal of all parathyroid tissue may have allowed another, more sluggish mechanism to come to light—for example, an alteration in the net calcium flux across the intestinal wall.

Whether a similar adapted state develops in humans deprived of all parathyroid tissue is uncertain. Mammalian parathyroid physiology, and the status of parathyroid grafted tissue in particular, should be reviewed in the light of these results.

## SUMMARY

1. Six normal dogs and ten adapted parathyroidectomized dogs have been studied with a view to defining the differences in calcium and phosphorus homeostasis in the two groups. The adapted animals required support during the acute hypocalcemic period after removal of their parathyroid glands for from 19 to 78 days. For the five months that these dogs were studied in the course of this experiment, no special support was required.

2. Parathyroidectomized dogs could not be distinguished with certainty from normal dogs either by clinical observation or by studies of serum calcium or phosphorus levels, phosphorus: creatinine clearance ratios after a calcium infusion, or tubular maximal reabsorption of phosphorus.

3. Parathyroidectomized dogs receiving intravenous infusions of either calcium gluconate (15 mg per kg in one hour) or EDTA (50 mg per kg in one hour) demonstrated markedly impaired ability to restore their serum calcium levels to the initial value, as compared with normal dogs. This response was unaltered by thyroid replacement therapy.

4. These findings imply that the parathyroid glands play an important part in dealing with acute disturbances of the serum calcium level. However, a secondary mechanism, capable of more sluggish regulation, seems to be revealed when the parathyroid glands are removed.

## ACKNOWLEDGMENT

The assistance of Winthrop Laboratories, Inc., and the Upjohn Company is gratefully acknowledged.

## REFERENCES


1. Wilson, R. E., Zollinger, R. M., Jr., Mahan, J. H., and Brooks, J. R. Homotransplantation of canine parathyroid tissue in millipore diffusion chambers: The phenomenon of adaptation to the aparathyroid state. Surg. Forum 1959, **10**, 94.
2. Munson, P. L., Iseri, O. A., Kenny, A. D., Cohn, V., and Sheps, M. C. A rapid and precise semimicro method for the analysis of calcium (abstract). J. dent. Res. 1955, **34**, 714.
3. Gomori, G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. clin. Med. 1942, **27**, 955.
4. Folin, O., and Wu, H. A system of blood analysis. J. biol. Chem. 1919, **38**, 81.
5. Owen, J. A., Iggo, B., Scandrett, F. J., and Stewart, C. P. Determination of creatinine in plasma or serum, and in urine; critical examination. Biochem. J. 1954, **58**, 426.
6. Hogben, C. A. M., and Bollman, J. L. Renal reabsorption of phosphate: Normal and thyroparathyroidectomized dog. Amer. J. Physiol. 1951, **164**, 670.
7. Thompson, D. D., and Hiatt, H. H. Renal reabsorption of phosphate in normal human subjects and in patients with parathyroid disease. J. clin. Invest. 1957, **36**, 550.
8. Howard, J. E., Hopkins, T. R., and Connor, T. B. On certain physiologic responses to intravenous injection of calcium salts into normal, hyperparathyroid and hypoparathyroid persons. J. clin. Endocr. 1953, **13**, 1.
9. Nordin, B. E. C., and Fraser, R. The effect of intravenous calcium on phosphate excretion. Clin. Sci. 1954, **13**, 477.
10. Goldman, R., and Bassett, S. H. Effect of I.V. calcium gluconate upon excretion of Ca and P in patients with idiopathic hypoparathyroidism. J. clin. Endocr. 1954, **14**, 278.
11. Patt, H. M., and Luckhardt, A. B. Relationship of a low blood calcium to parathyroid secretion. Endocrinology 1942, **31**, 384.
12. Stewart, G. S., and Bowen, H. F. The parathyroid control of serum calcium independent of renal mediation. Endocrinology 1951, **48**, 568.
13. Copp, D. H. Calcium and phosphorus metabolism. Amer. J. Med. 1957, **22**, 275.
14. Talmage, R. V. Studies on the maintenance of serum calcium levels by parathyroid action on bone and kidney. Ann. N. Y. Acad. Sci. 1956, **64**, 326.
15. Kleeman, C. R., Rockney, R. E., and Maxwell, M. H. The effect of parathyroid extract (PTE) on the renal clearance of diffusible calcium (abstract). J. clin. Invest. 1958, **37**, 907.
16. Barnicot, N. A. The local action of parathyroid and other tissues on bone in intracerebral grafts. J. Anat. (Lond.) 1948, **82**, 233.
17. Connor, T. B., Thomas, W. C., Jr., and Howard, J. E. The etiology of hypercalcemia associated with lung carcinoma (abstract). J. clin. Invest. 1956, **35**, 697.
18. Schatten, W. E., Ship, A. G., Pieper, W. J., and Bartter, F. C. Syndrome resembling hyperparathyroidism associated with squamous cell carcinoma. Ann. Surg. 1958, **148**, 890.

# LOCALIZATION OF LEUCINE AMINOPEPTIDASE IN SERUM AND BODY FLUIDS BY STARCH GEL ELECTROPHORESIS *

By O. DHODANAND KOWLESSAR, LORRAINE J. HAEFFNER AND MARVIN H. SLEISENGER

*(From the Department of Medicine, New York Hospital-Cornell Medical Center, New York, N. Y.)*

(Submitted for publication October 9, 1959; accepted December 10, 1959)

Serum leucine aminopeptidase (LAP), as measured by the method of Goldbarg and Rutenburg (1), is elevated in patients with biliary cirrhosis, intrahepatic cholestasis, metastatic carcinoma of the liver, carcinoma of the pancreas obstructing the common bile duct or metastatic to the liver, carcinoma of the biliary tree, primary hepatoma, infiltrative diseases of the liver such as lymphoma, sarcoidosis, and so forth, and infectious hepatitis (2, 3).

In order further to compare serum LAP activity in normal subjects and in patients with a variety of diseases involving the pancreas, liver and biliary tree, its mobility was measured by starch gel electrophoresis. Although there was a common peak of activity in the sera of both normal subjects and of patients with the diseases studied, LAP was also noted at other sites in the latter group.

## MATERIALS AND METHODS

All sera were obtained from fasting, normal healthy subjects and from patients on the medical and surgical wards of The New York Hospital, whose diagnoses were confirmed either by biopsy, surgical exploration, autopsy, or by their clinical courses.

The study group included 8 normal adults and 36 patients with a variety of inflammatory, malignant, and granulomatous diseases affecting the liver, biliary tract, and/or the pancreas. There were 6 patients with primary biliary cirrhosis; 6 with acute infectious hepatitis; 5 with Laennec's cirrhosis and acute fatty infiltration of the liver; 1 with lymphosarcoma involving the liver; 1 with sarcoidosis; 2 with Hodgkin's disease; 4 with carcinoma of the colon, metastatic to the liver; 2 with common duct stone; 1 with toxic hepatitis secondary to Mitomycin; and 1 with infectious mononucleosis. Seven patients with carcinoma of the pancreas were also studied (Table I). In 6 the tumor was in the head of the pancreas. Of these, 2 had obstruction of the common bile duct but no metastases to the liver; 2 had no obstruction but had hepatic metastases; and 2 had neither obstruction nor metastases. One patient's lesion was in the body and tail of the pancreas but was metastatic to the liver. This individual, however, had no common duct obstruction.

All of the patients had elevated levels of serum LAP, except the two with carcinoma of the head of the pancreas with neither common duct obstruction nor metastases to the liver. Three patients with viral hepatitis and two with cirrhosis and fatty infiltration were studied serially and upon recovery, when the elevated serum LAP had fallen to normal.

Serum proteins were separated by zone electrophoresis according to a modification of the starch gel method of Smithies (4), reported by Pert, Sleisenger, Woods and Engle (5). Electrophoresis was carried out at 12° C at 4.5 v per cm for 16 hours. In early experiments electrophoresis was performed using starch gel strips 2 cm wide. After completion of the electrophoretic run, the gels were scored in 0.5 cm sections and cut in half along the horizontal plane. One half was retained for protein staining with a saturated solution of naphthalene black 12B 200 (4), and the second half was cut in 0.5 cm sections which were placed directly in tubes containing the necessary amount of buffered substrate for the determination of LAP by the method of Goldbarg and Rutenburg, at pH 7.1 utilizing a chromogenic substrate, L-leucyl-β-naphthylamide hydrochloride. The β-naphthylamine liberated by enzymatic hydrolysis was assayed by a modified Bratton-Marshall reaction (1). Activity under each major peak was expressed as percentage of the sum total of all 0.5 cm sections of the gel strips (Table I).

In later experiments, when information was needed regarding the amount of protein present in each 0.5 cm section, starch gel strips, 4.5 cm wide, were used. The serum protein was separated by zone electrophoresis in a similar manner. The strips were scored in 0.5 cm sections but were divided in half vertically before being halved along the horizontal plane. One-fourth of the gel was retained for protein staining. In the remaining three-fourths, corresponding 0.5 cm sections of the gel were placed in separate tubes containing 3 ml of water, mechanically shaken for 30 minutes at room temperature, and eluates of each 0.5 cm section were recovered. The eluates were then filtered and 0.2 ml of the filtrate was assayed for protein by the micromethod of Lowry,

* This work was supported by Research Grant CS-9386 from the National Institutes of Health, Bethesda, Md.

TABLE I

*Electrophoretic distribution of leucine aminopeptidase (LAP) in serum and body fluids*

| Diagnosis | No. studied | No. of peaks of LAP found | Mean % activity and standard deviation of mean in each peak* | | |
|---|---|---|---|---|---|
| | | | Postalbumin or fast $\alpha_2$ to postalbumin | Origin to slow $\alpha_2$ | $\alpha_2$ to $\beta$-globulin |
| Normal subjects | 8 | 1 | 67.5 ± 2.2 | 0 | 0 |
| Biliary cirrhosis | 6 | 2 | 68.0 ± 1.4 | 11 ± 2.0 | 0 |
| Toxic hepatitis secondary to Mitomycin | 1 | 2 | 55.0 | 30 | 0 |
| Acute infectious hepatitis | 6 | 3 | 57.0 ± 2.5 | 9.5 ± 1.0 | 14 ± 0.5 |
| Infectious hepatitis (recovered) | 3 | 1 | 66.0 ± 3.4 | 0 | 0 |
| Laennec's cirrhosis with acute fatty infiltration | 5 | 3 | 58.0 ± 1.8 | 8 ± 0.4 | 20 ± 0.6 |
| Laennec's cirrhosis after recovery | 2 | 1 | 68.0 | 0 | 0 |
| Lymphoma | 1 | 3 | 68.0 | 5 | 14 |
| Hodgkin's disease | 2 | 3 | 70.0 | 5 | 12 |
| Sarcoidosis | 1 | 3 | 67.0 | 5 | 14 |
| Common duct stone | 2 | 3 | 45.0 | 15 | 15 |
| Carcinoma pancreas with elevated LAP† | 5 | 3 | 48.0 ± 0.4 | 15 ± 0.5 | 15 ± 1.2 |
| Carcinoma of head of pancreas with normal LAP‡ | 2 | 1 | 65.0 | 0 | 0 |
| Metastatic carcinoma to liver | 4 | 3 | 45.0 ± 2.1 | 15 ± 1.1 | 12 ± 1.3 |
| Infectious mononucleosis | 1 | 3 | 45 | 12 | 20 |
| Bile | 3 | 1 | 80 ± 1§ | 0 | 0 |
| Ascitic fluid | 1 | 1 | 80§ | 0 | 0 |
| Pleural fluid | 1 | 1 | 82§ | 0 | 0 |

* Mean per cent and standard deviation of the mean per cent when more than two specimens were studied.
† Tumors had either obstructed common bile duct or metastasized to liver.
‡ Tumors neither obstructed common duct nor metastasized to liver.
§ Specimens were diluted 1:1 with normal serum.

Rosebrough, Farr and Randall (6), and compared with a bovine albumin standard. One ml of the filtrate was used for the determination of LAP.

LAP activity is plotted against the distances per 0.5 cm migrated (cross-hatched bars; Figures 1–3, 6). Protein concentrations of the filtered eluate are similarly plotted for each 0.5 cm section (open bars; Figures 4, 5). In all figures, except B in Figure 1, electrophoregrams are placed on the abscissa to locate peak LAP activity in terms of the component protein fractions.

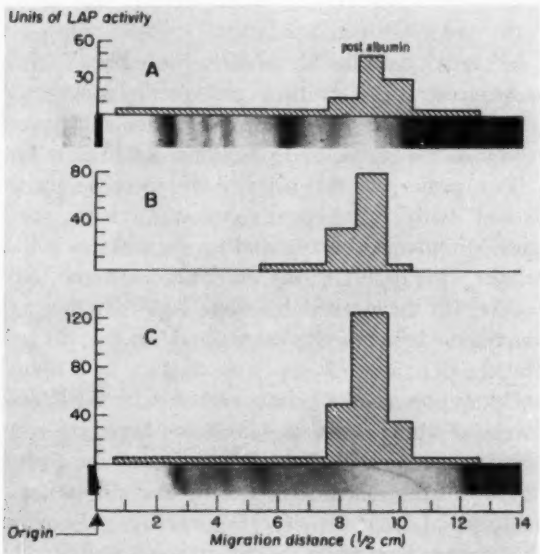
Similarly, electrophoresis, protein assays, and LAP determinations were performed on specimens of ascitic fluid, pleural fluid, bile obtained from patients by T-tube drainage of the common duct and by peroral duodenal intubation, 1 per cent pancreatic extract (Organon), and mixtures of the latter two substances with normal and abnormal sera.

## RESULTS

The starch gel electrophoretic patterns showed that in all instances the major zone of LAP activity coincided exactly with the postalbumin fraction of serum or lay in the region of the fast $\alpha_2$ and postalbumin fractions. In 34 pathological sera there was significant activity in the region between the origin and slow $\alpha_2$, and in 27 of these specimens a peak of activity was also found in the region of the slow $\alpha_2$ and $\beta$-globulin fractions. The area labeled origin to slow $\alpha_2$ does not stain with the protein stain used in our experiments, but it can be seen that there is a large amount of protein present as measured by the microprotein determination (Figures 4, 5).

Table I summarizes the results obtained from 54 specimens and includes the diagnosis, the nature of specimen studied, the number of peaks of LAP found and the activity of each peak expressed as percentage of the sum total of all 0.5 cm sections of the gel.

*Normal subjects.* In the sera of all eight normal subjects, there was one peak of LAP activity. In six specimens this peak coincided exactly with the postalbumin fraction of serum, but in two instances it lay in the region of the fast $\alpha_2$ and postalbumin (Figure 1). In no instance was any other peak of LAP activity found, even though there was measurable activity in several segments of the gel. No LAP activity was present in the gamma globulin and albumin fractions.



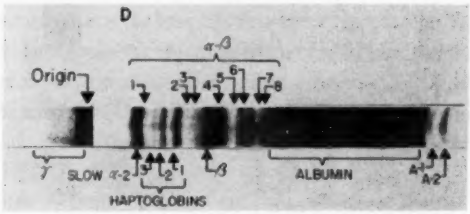




FIG. 1. LOCALIZATION OF LEUCINE AMINOPEPTIDASE (LAP) BY STARCH GEL ELECTROPHORESIS, DEMONSTRATING PEAKS OF ENZYME ACTIVITY. A. Normal serum. B. Bile. C. Bile and normal serum mixture (1:1). A stained electrophoregram was not obtained when bile was electrophoresed, due to the small amount of protein present in bile. D. Starch gel electrophoregram of normal serum for reference. In $\alpha$–$\beta$ complex, fraction 6 = fast $\alpha_2$, and fractions 7 and 8 = postalbumin.

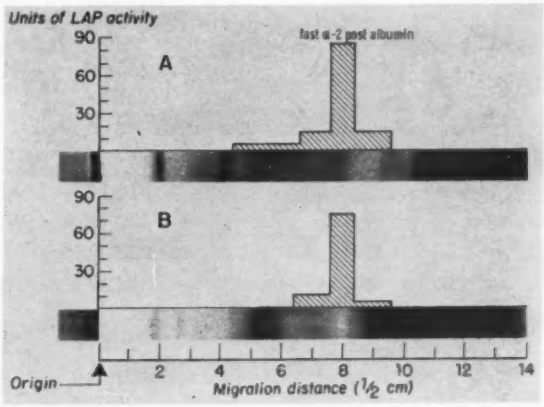



FIG. 2. LOCALIZATION OF LAP IN BODY FLUIDS BY STARCH GEL ELECTROPHORESIS. A. Ascitic fluid from a patient with Laennec's cirrhosis. B. Pleural fluid from a patient with carcinoma of the lung.

*Biological fluids.* Bile, obtained from both choledochostomy drainage and peroral duodenal intubation, pleural fluid, ascitic fluid, and pancreatic extract (Organon) run separately or diluted 1:1 with normal sera, also demonstrated only one peak of LAP activity, coinciding with the postalbumin fraction or the fast $\alpha_2$ and postalbumin (Figures 1, 2). When these fluids were diluted with sera having elevated LAP activity, all peaks of LAP activity were preserved and the postalbumin peak or the peak coinciding with the fast $\alpha_2$ and postalbumin was augmented.

*Pathological sera.* In all six cases of *acute infectious hepatitis,* three peaks of LAP activity were found. An average of 57 per cent of the total enzyme activity either coincided with the

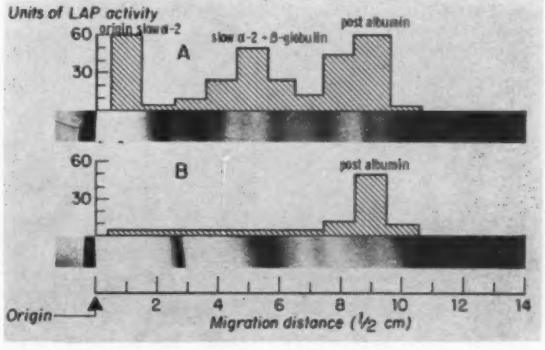



FIG. 3. LOCALIZATION OF SERUM LAP IN INFECTIOUS HEPATITIS. A. During acute phase of disease—third day. B. During recovery, when serum LAP and liver biopsy were normal.

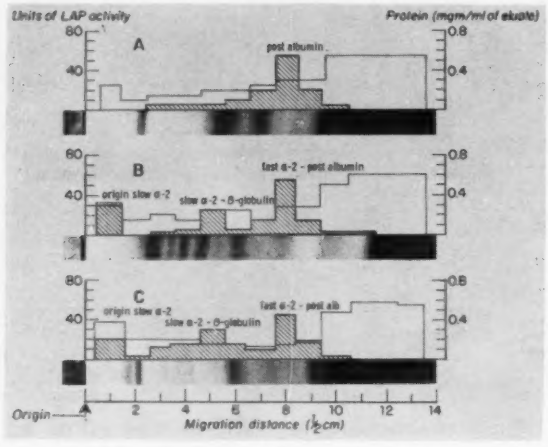



FIG. 4. LOCALIZATION OF SERUM LAP IN CARCINOMA. A. Patient with carcinoma of the pancreas with normal serum LAP. B. Patient with carcinoma of the pancreas metastatic to the liver with elevated serum LAP. C. Patient with carcinoma of the cecum metastatic to the liver. Open bar = protein (milligrams per milliliter of eluate). Hatched bar = units of LAP activity.

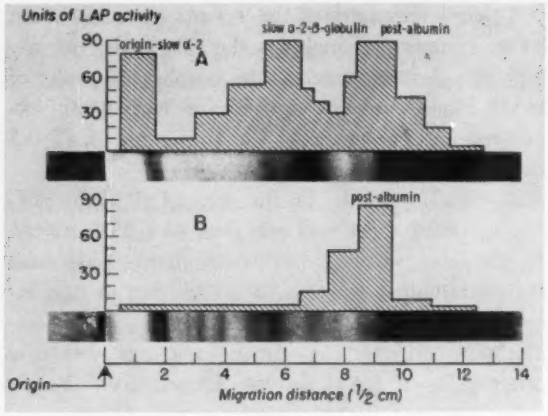



FIG. 6. LOCALIZATION OF SERUM LAP IN COMMON BILE DUCT OBSTRUCTION. A. Patient with common bile duct stone. B. Thirty days after surgical relief of the obstruction.

postalbumin fraction or was in its immediate vicinity. The average activity in the origin and slow $\alpha_2$ fraction and in the region of slow $\alpha_2$ and $\beta$-globulin fractions was 9.5 and 14 per cent, respectively (Figure 3). *Infiltrative diseases of the liver,* i.e., sarcoidosis, lymphosarcoma, and Hodgkin's disease, *carcinoma of the pancreas* with obstruction of the common bile duct or metastatic to the liver and elevated serum LAP; *common bile duct stone; Laennec's cirrhosis* with acute fatty infiltration; and *metastatic carcinoma of the liver* all consistently showed three peaks of

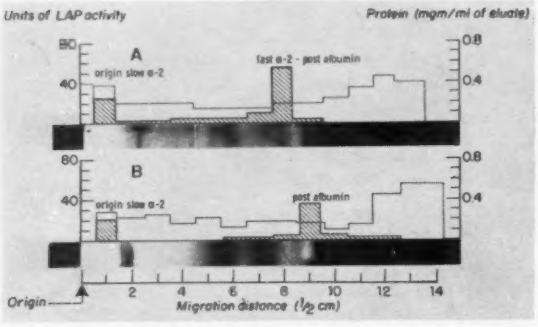



FIG. 5. LOCALIZATION OF SERUM LAP IN INTRAHEPATIC CHOLESTASIS. A. Patient with biliary cirrhosis. B. Patient with cholangiolitic hepatitis secondary to Mitomycin.

LAP activity similar to those in the patients with acute infectious hepatitis, the predominant activity lying in the postalbumin fraction or in the region of the fast $\alpha_2$ and postalbumin (Figure 4).

Two peaks of LAP activity were noted in one patient with intrahepatic cholestasis associated with Mitomycin therapy and in six patients with primary biliary cirrhosis, the predominant one residing in the normal fractions and the other in the region between the origin and slow $\alpha_2$ (Figure 5).

Upon complete clinical and biochemical recovery of three patients with acute hepatitis and of two patients with fatty infiltration of the liver, repeat studies demonstrated only the single normal peak of LAP activity (Figure 3). A similar observation was made in two patients with common duct stone, who demonstrated a single normal peak of LAP activity after removal of the calculus (Figure 6). The disappearance of two peaks upon cure of these conditions also coincided with the fall of serum LAP to normal.

## DISCUSSION

Leucine aminopeptidase activity of normal serum, measured at pH 7.1, appears as a single peak with an electrophoretic mobility on starch gel which coincides with the postalbumin fraction of serum or the fast $\alpha_2$ and postalbumin fractions. Bile, pancreatic extract (Organon), and mixtures

of these substances with normal serum likewise demonstrated a single peak of LAP activity similar to that found in normal serum.

The similarity of electrophoretic mobility of LAP from serum and bile and the histochemical localization of LAP in bile canaliculi (7) strongly suggest that these structures are the source of both serum and bile LAP which migrate with the postalbumin fraction or the fast $\alpha_2$ and postalbumin fractions. The possibility that the reverse is true, namely, that bile LAP is derived from serum, cannot be excluded.

The presence of two additional peaks of LAP activity in patients with either parenchymal or metastatic liver disease and their disappearance from the sera of those patients who have had clinical and biochemical recovery from infectious hepatitis and fatty infiltration of the liver is consistent with the possibility that LAP in the regions of origin to slow $\alpha_2$ and $\alpha_2$ to $\beta$-globulin is derived from the liver. However, their disappearance following relief of common bile duct obstruction, leaving the single normal peak, also indicates that stasis may alter LAP.

It is noteworthy that the electrophoretic mobility of serum LAP in two patients with carcinoma of the head of the pancreas was similar to that in normal subjects. Neither patient had jaundice or metastasis to the liver, and the total serum LAP activity in each was normal. In contrast, in patients with carcinoma of the head or body and tail of the pancreas who had either common duct obstruction or metastases to the liver, the serum LAP was elevated. Further, such patients demonstrated three peaks of LAP activity by starch gel electrophoresis. This finding is not specific for carcinoma of the pancreas since it was observed in four patients with carcinoma of the colon with metastases to the liver and in two patients with choledocholithiasis (Figures 4, 6).

These findings suggest that neither elevation nor electrophoretic alteration of serum LAP is specific for carcinoma of the pancreas; rather they support the hypothesis that such changes are dependent upon common bile duct obstruction or metastasis to the liver.

SUMMARY

1. The electrophoretic mobility of leucine aminopeptidase (LAP) of normal serum, sera of patients with pancreatic and hepatobiliary disease, bile, pleural fluid and pancreatic extract has been determined by starch gel electrophoresis.

2. There is a single peak of activity in normal serum identical with that of bile and pancreatic extract, located in the postalbumin fraction or in the fast $\alpha_2$ and postalbumin areas.

3. Two additional peaks of LAP activity are found in the sera of patients with parenchymal or metastatic liver disease and with common bile duct obstruction. With recovery of liver disease or relief of obstruction, these peaks disappear.

4. Electrophoretic mobility of serum LAP in patients with carcinoma of the pancreas is normal, unless there is common duct obstruction or metastasis to the liver.

ACKNOWLEDGMENT

The authors wish to acknowledge the assistance of Dr. Graham Jefferies and Mrs. Joan Freudenberger in the performance of the starch gel electrophoresis.

REFERENCES

1. Goldbarg, J. A., and Rutenburg, A. M. Colorimetric determination of leucine aminopeptidase in urine and serum of normal subjects and patients with cancer and other diseases. Cancer 1958, **11**, 283.
2. Kowlessar, O. D., Haeffner, L. J., and Sleisenger, M. H. To be published.
3. Rutenburg, A. M., Goldbarg, J. A., and Pineda, E. P. Leucine aminopeptidase activity. Observations in patients with cancer of the pancreas and other diseases. New Engl. J. Med. 1958, **259**, 469.
4. Smithies, O. Zone electrophoresis in starch gels: Group variations in the serum proteins of normal human adults. Biochem. J. 1955, **61**, 629.
5. Pert, J. H., Sleisenger, M. H., Woods, K. R., and Engle, R. L., Jr. Requirements for optimal resolving power and reproducibility in protein fractionation by starch gel electrophoresis. Clin. Res. Proc. 1957, **5**, 156.
6. Lowry, O. H., Rosebrough, M. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. biol. Chem. 1951, **193**, 265.
7. Nachlas, M. N., Crawford, D. T., and Seligman, A. M. Histochemical demonstration of leucine aminopeptidase. J. Histochem. Cytochem. 1957, **5**, 264.

# HISTAMINE METABOLISM IN HUMAN DISEASE * †

By GILDON N. BEALL ‡ and PAUL P. VANARSDEL, Jr.

*(From the Department of Medicine, University of Washington School of Medicine, Seattle, Wash.)*

(Submitted for publication October 27, 1959; accepted December 10, 1959)

The mode of histamine catabolism in various experimental animals has been carefully studied in recent years. Such studies have been facilitated by the availability of ring-labeled histamine-$C^{14}$. Two main metabolic pathways have been found (Figure 1). In one, histamine is oxidized to imidazole-4(5)-acetic acid (ImAA) through imidazole-4(5)-acetaldehyde. This reaction *in vitro* can be catalyzed by a diamine oxidase found in kidney tissue of most species. A xanthine oxidase or an aldehyde dehydrogenase is needed to complete the reaction. Much of the ImAA is then conjugated with ribose and excreted in the urine as such (1).

A second pathway proceeds through methylation of the ring imino-nitrogen to form 1,4-methylhistamine followed by oxidation of the side chain, presumably through the corresponding aldehyde. *In vitro* reproduction of the methylation step has recently been accomplished with an imidazole-N-methyl transferase found in many tissues (2–5). The oxidation is catalyzed by a monoamine oxidase found in mouse and cat liver and in human kidney. Most of the radioactivity administered as histamine is excreted as 1,4-methylimidazoleacetic acid (meImAA) by mice and cats.

Studies on histamine-$C^{14}$ metabolism in humans have been published recently by Schayer and Cooper (6) and Nilsson, Lindell, Schayer and Westling (7), and indicate that both pathways of catabolism are involved, with the principal metabolites being meImAA and ImAA (free and conjugated). In pregnancy, little alteration in this pattern occurred; no disease states were reported.

Abnormalities of histamine catabolism have been suspected in several diseases. Investigations designed to discover such abnormalities have not been particularly successful (8–13). Failure in some cases can be attributed to inappropriate measurements and in others to inadequate methods. The assay of whole blood histamine, for instance, reflects mainly the amount of histamine contained in the leukocytes, although assays on plasma should be more appropriate for determining the rate of release or catabolism of histamine. Studies on histamine excreted in the urine have given contradictory results; such assays are misleading and of questionable value since less than 5 per cent of intravenously or subcutaneously administered histamine is excreted unchanged (6, 14).

The objectives of this study were: *1*) to establish the normal pattern of metabolism of exogenous histamine in man; *2*) to look for an altered pattern of metabolism in disorders (asthma, histaminic cephalalgia) in which an altered reactivity to histamine is suspected clinically; and *3*) to determine the pattern of metabolism in patients with insufficient liver or kidney function which conceivably could involve histamine-metabolizing enzymes.

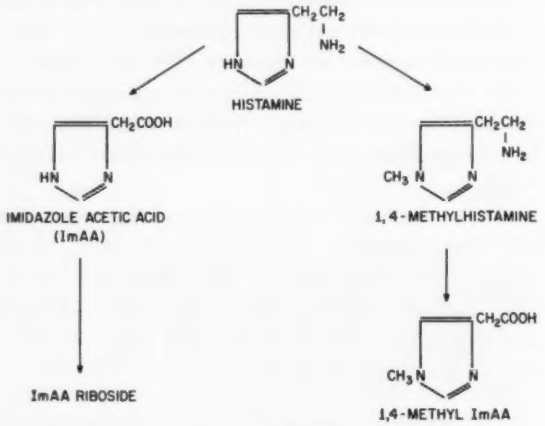



Fig. 1. Pathways of histamine catabolism.

* Supported by Grant E-1510 of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.

† Presented in part before the Western Section of the American Federation for Clinical Research at Carmel, Calif., January 28, 1959.

‡ Daland Fellow of the American Philosophical Society, 1957–1959.

## METHODS AND MATERIALS

Nineteen subjects participated in the study. There were 3 with bronchial asthma, 1 with uremia from chronic glomerulonephritis, 5 with chronic liver insufficiency, and 3 with histaminic cephalalgia. Seven control subjects included 2 healthy young men and 5 convalescent patients with presumably unrelated diseases. The patients with Laennec's cirrhosis had far-advanced disease with parenchymal damage and jaundice. Three of the 5 were dead within a year after the study. The 3 patients with histaminic cephalalgia had typical attacks of unilateral throbbing headache, usually associated with ipsilateral nasal congestion and lacrimation. Two of the patients had previously had attempts at histamine "desensitization" with equivocal results.

The histamine-$C^{14}$, labeled in the 2 position of the ring, had a specific activity of 4.52 mc per mmole.[1] Fifty to 100 $\mu$g was injected intravenously in 30 seconds. Samples of venous blood were removed at intervals from 1 minute to 48 hours after the injection, and all urine was collected for at least 48 hours. Radioactivity of the plasma and urine samples was assayed with a liquid scintillation spectrometer by methods previously described (15). Samples containing more than 70 disintegrations per minute were counted long enough to provide an accuracy of $\pm 5$ per cent at the 0.9 confidence limit. Samples with less than 70 disintegrations per minute were counted for 1 hour. The statistical limits were obtained from the nomograms of Jarrett (16).

The amount of unchanged histamine-$C^{14}$ in plasma was determined by subjecting some of the plasma samples to the concentrating and extracting steps of the chemical assay for histamine developed by Lowry and colleagues (17), followed by radioactive assay of the final extract. The remainder of the radioactivity was assumed to represent histamine metabolites.[2] The plasma radioactivity was concentrated 50- to 100-fold by this extraction procedure, thus providing sufficient radioactivity in the very small volume required for liquid scintillation assays (15).

Histamine-$C^{14}$ metabolism in a normal subject was studied during periods of administration of a potent antihistaminic drug, chlorprophenpyridamine, and a histamine analog, 3-$\beta$-aminoethylpyrazole.[3] The chlorprophenpyridamine was given in doses of 8 mg every 4 hours, starting 12 hours before the study. The 3-$\beta$-aminoethylpyrazole was given subcutaneously in 3 doses of 50 mg 2 hours before, immediately after and 2 hours after the injection of histamine-$C^{14}$.

One subject, GH, with histaminic cephalalgia, had his studies repeated after "desensitization" to histamine. The "desensitization" was carried out by giving histamine subcutaneously 3 times per day for 8 days, increasing the dosage to a maximum of 200 mg.

Separation of the radioactive metabolites in the urine was accomplished by paper chromatography. A 10 or 20 ml aliquot of urine, voided during the first 3 hours after injection, was dried and extracted with methanol in a Soxhlet apparatus for 2 hours. A few samples obtained at later times were similarly treated. The methanol extract was concentrated *in vacuo* to a volume of 5 ml or less and the resulting precipitate discarded. The concentrated methanol solution was found to contain about 90 per cent of the radioactivity originally present in the urine specimen. (The range was 76 to 108 per cent in 18 extractions.)

Two-dimensional ascending paper chromatography was performed at room temperature with Whatman no. 1 paper (19 cm square). In one corner, 2 cm from either edge, 0.02 to 0.5 ml of the concentrated methanol extract was placed on the paper. Initial development was with *n*-butanol, concentrated $NH_4OH$, and 95 per cent ethanol in proportions of 8:3:1 (1). After the paper was rotated 90°, development was performed with *n*-butanol, glacial acetic acid, and water in proportions of 4:1:5. We found that two development "passes" of 3 to 4 hours each in each direction (with drying of the paper between each run) provided optimal separation of the radioactive areas.

Histamine-$C^{14}$ metabolites were detected by exposing Kodak no-screen X-ray film to the chromatogram for at least 2 weeks. Some chromatograms were exposed for as long as 3 months. Nonlabeled substances were used to aid identification of the radioactive metabolites. Histamine dihydrochloride, ImAA, and acetylhistamine were detected by spraying chromatograms with diazotized sulfanilic acid (19). Brom-cresol green (20) was used to detect spots of 1,4-meImAA.[4] Quantitation of the radioactive areas was attempted by elution with water followed by assay of the eluate with the liquid scintillation spectrometer.

## RESULTS

Histamine-$C^{14}$ was injected intravenously 23 times in 19 subjects. Ten to 15 seconds after the

[1] Obtained from the Nuclear-Chicago Corp., Chicago 10, Ill.

[2] Acetylhistamine and other derivatives of histamine with substitutions in the amino group will not behave as histamine in this procedure (17). Of the various histamine metabolites found by Schayer and Tabor, the only substance with a free amino group is 1,4-methylhistamine. We have not ruled out the possibility that this may mimic histamine in the chemical determination.

[3] Histalog; generously supplied by Eli Lilly & Co., Indianapolis, Ind. (18).

[4] Acetylhistamine was generously provided by Dr. H. Tabor, National Institutes of Health, Bethesda, Md. The 1,4-meImAA was generously donated by Dr. R. W. Schayer, Merck Institute for Therapeutic Research, Rahway, N. J. Histamine dihydrochloride was purchased from Eastman Organic Chemicals, Rochester, N. Y. ImAA (as the hydrochloride) was purchased from K & K Laboratories, Long Island City, N. Y.

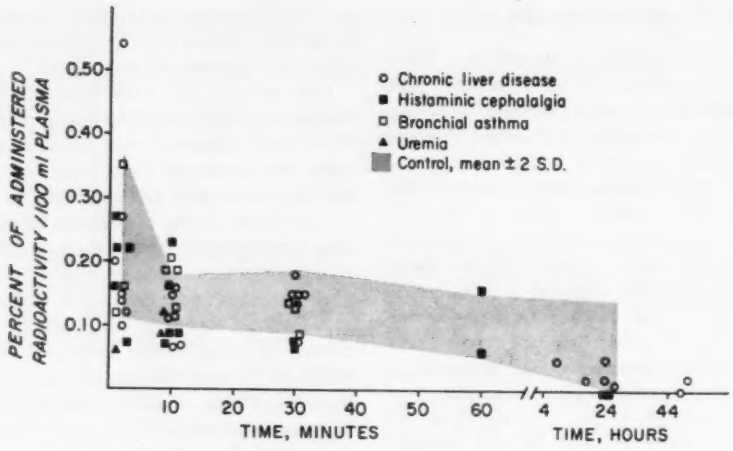



FIG. 2. TOTAL PLASMA RADIOACTIVITY EXPRESSED AS PERCENTAGE OF ADMINISTERED RADIOACTIVITY.

injection had begun most persons developed a flush, tachycardia, and mild hypotension. The duration and degree of these responses varied directly with the amount of histamine given; little clinical response occurred with less than 60 $\mu$g. Blood pressure began to rise again 50 to 60 seconds after the injection had begun, at which time the pounding occipital headache usually started. All the symptoms but headache were gone three minutes after the injection. As expected, each of the three asthmatic patients experienced mild bronchospasm for about five minutes following histamine injection (21). Although histamine injection is known to provoke typical attacks of histaminic cephalalgia (22), none of our three patients had such a reaction.

*A. Studies on plasma.* Plasma radioactivity was expressed as the fraction (per cent) of the administered radioactivity present in 100 ml of plasma, and the values are presented in Figure 2. The mean of all samples obtained during the first three and one-half minutes after the injection was 0.24 per cent of the administered dose per 100 ml plasma ($\pm$ 0.02 at the 0.9 confidence limit). The concentration of radioactivity in the plasma decreased rapidly with the passage of time although a trace was present for many hours. No difference in the patterns of various groups emerged.

Not only was the radioactivity cleared rapidly from the plasma, but the plasma radioactivity that was present was largely due to metabolites of histamine-$C^{14}$ and not to histamine-$C^{14}$ itself. Figure 3 compares the means of the points shown in Figure 2 with the means for true histamine-$C^{14}$. Although some radioactivity persisted in the plasma for many hours, the "true" histamine radioactivity disappeared from the plasma promptly.

*B. Urinary excretion.* The radioactivity was excreted rapidly into the urine of all subjects except Patient AS. In most cases, 70 per cent or more of the administered radioactivity was excreted in 24 hours. Excretion was essentially complete in 30 hours.

Figure 4 shows the cumulative recovery of the

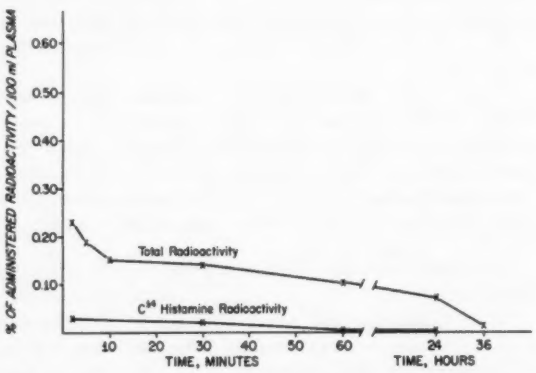



FIG. 3. MEANS OF DATA PLOTTED IN FIGURE 2 COMPARED WITH MEANS OF SPECIFIC HISTAMINE-$C^{14}$ RADIOACTIVITY IN PLASMA. An initial level of at least 0.5 per cent per 100 ml would be expected if the administered histamine were rapidly distributed in extracellular fluid, without being taken up by cells.

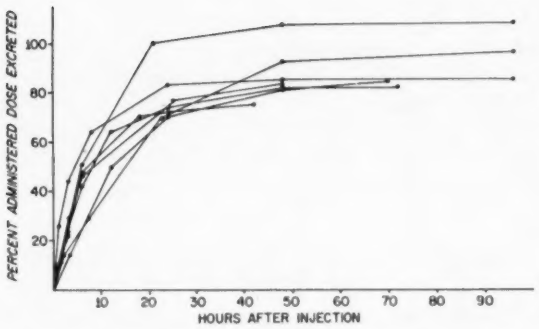



FIG. 4. CUMULATIVE RECOVERY OF ADMINISTERED RADIOACTIVITY FROM URINE OF SEVEN CONTROL SUBJECTS.

administered dose from the control subjects. Figure 5 shows the same data for the subjects with specific disorders. There was considerable variation in the initial rate of excretion. The urine collected from one asthmatic and two cirrhotic subjects contained only about 60 per cent of the injected radioactivity (Figure 5). Since the initial rate of excretion was rapid in all three of these subjects, the failure to recover more radioactivity was attributed to incomplete urine collection rather than to any metabolic abnormality. The patient with uremia and renal insufficiency (AS) excreted the radioactvity at a slow constant rate for at least 12 days.

*C. Chromatography.* Radioautography of the two-dimensional chromatograms of the control urines revealed three distinct radioactive areas (Figure 6). A fourth area was detected in the urine chromatograms of two of the five subjects. These were usually 3 to 4 sq cm in area and irregular in shape. The $R_F$ values for each area

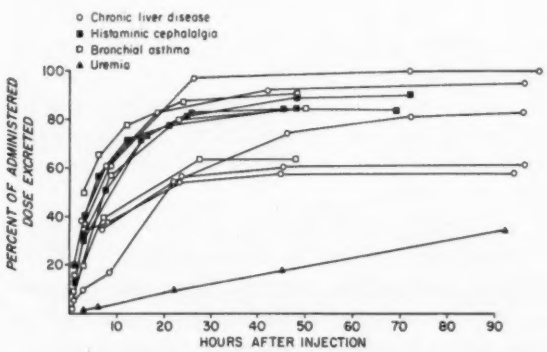



FIG. 5. CUMULATIVE RECOVERY OF ADMINISTERED RADIOACTIVITY FROM URINE OF SUBJECTS WITH VARIOUS DISEASES.

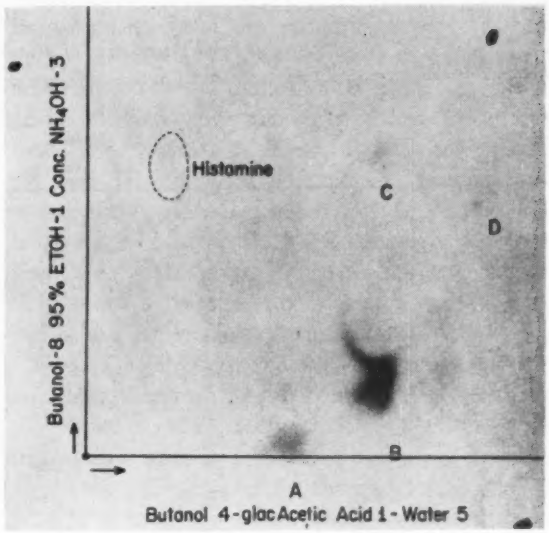



FIG. 6. TWO-DIMENSIONAL CHROMATOGRAM OF URINE EXTRACT. Three distinct areas are marked A, B and C. A fourth spot, D, was occasionally found. The dotted area indicates the locus of histamine when chromatographed in this fashion.

varied somewhat among the chromatograms, probably due to the varying amounts of interfering substances present in the concentrated methanol extracts of different urines. However, the darkened areas found were always similarly placed on the film in relation to each other. Elution of the radioactive areas followed by repeat chromatography of the eluate established the individuality of the three different substances and the identity of similar spots from different chromatograms. When eluates from spot B of two different chromatograms were combined with nonradioactive ImAA and subjected to chromatography, identical migration occurred ($R_F$ 0.21 in solvent 1 and 0.57 in solvent 2). This was regarded as reliable evidence that spot B was ImAA. Spot A was identified as ImAA-riboside on the basis of $R_F$ values and chromatographic similarity to rat ImAA-riboside.[5] Spot C was identified as me-ImAA by comparison with migration of the nonlabeled substance, but our efforts to elute enough of the radioactive material from a chromatogram for direct comparison of the mixture were un-

[5] The latter was obtained by elution from a paper chromatogram of urine from a rat given histamine-$C^{14}$ intravenously. It is the major metabolite in rat urine (23).

TABLE I

*Recovery and distribution of urinary radioactivity after chromatography*

| | Amount elutable | Distribution | | | |
|---|---|---|---|---|---|
| | | A ImAA-* riboside | B ImAA | C 1,4-Methyl-ImAA | D 1,4-Methyl-histamine |
| | % | % | % | % | % |
| Controls | | | | | |
| KT | 59 | 15 | 38 | 47 | |
| CB | 63 | 10 | 84 | 6 | |
| RT | 47 | 49 | 49 | 2 | |
| JC | 42 | 19 | 38 | 38 | 5 |
| SN | 60 | 45 | 35 | 8 | 12 |
| Mean | 54 | 28 | 49 | 20 | |
| Cirrhotics† | | | | | |
| RS | 56 | 25 | 65 | 6 | 4 |
| JF | 75 | 23 | 63 | 9 | 5 |
| Mean | 66 | 24 | 64 | 6 | 5 |
| Asthmatics | | | | | |
| JL | 90 | 17 | 63 | 20 | |
| EV | 39 | 33 | 62 | 5 | |
| HL | 44 | 27 | 66 | 7 | |
| Mean | 58 | 25 | 64 | 11 | |
| Histamine cephalalgia | | | | | |
| JW | 41 | 46 | 49 | 5 | |
| RB | 75 | 21 | 19 | 60 | |
| GH | 50 | 30 | 58 | 12 | |
| Mean | 56 | 32 | 42 | 26 | |
| Uremia | | | | | |
| AS | 63 | 100 | | | |

* Imidazole-4(5)-acetic acid.

† Because of interfering substances, chromatographic patterns adequate for elution and quantitation could not be obtained for the other three patients.

successful. Similarly, unknown D could be identified as 1,4-methyl histamine only by comparison of $R_F$ values. Chromatography and staining of nonlabeled histamine and acetylhistamine demonstrated that these substances moved to sites quite far removed from the radioactive areas.

Chromatography of urine samples collected at 6 and 12 hours was also carried out for two normal subjects and for one with cirrhosis. There were no differences in the metabolite distribution. Further studies were not done because the concentrations of radioactivity usually became too low for satisfactory chromatograms to be developed.

Table I illustrates the amounts of each of these metabolites that could be recovered from the paper chromatograms. In almost every case, ImAA was the principal metabolite. No consistent variation in the types or amounts of metabolites excreted was found between the several groups studied. The chromatogram from the subject with uremia was interesting in that only one radioactive area was found. This was located at the site to which ImAA-riboside usually migrated. However, the low concentration of radioactivity in his urine made it difficult to confirm or amplify this finding.

No significant changes in plasma or urinary radioactivity, or in the chromatography of the urinary metabolites occurred after antihistamine or 3-$\beta$-aminoethylpyrazole administration to Subject KT. Similarly, histamine "desensitization" did not alter the pattern of metabolism by Patient GH.

## DISCUSSION

The clearance of radioactive histamine from plasma was extremely rapid in all cases studied, and no differences among the various disease groups could be detected. The rate of urinary excretion of radioactive metabolites was also surprisingly constant in our studies. Only in the uremic patient was there an abnormality in urinary excretion; the radioactivity was excreted slowly at a relatively constant rate for at least 12 days. Since plasma levels of histamine-$C^{14}$ did not differ from the others, the deficiency in excretion of the radioactivity was probably due to impaired renal clearance rather than to a decrease in histamine-metabolizing activity.

Failure to find specific abnormalities in the overall rate of histamine-$C^{14}$ metabolism prompted us to search for more subtle alterations by paper chromatography of the urine. Imidazole-4(5)-acetic acid, ImAA-riboside and 1,4-meImAA were identified in the urine of all persons given histamine-$C^{14}$ except for the one with uremia, who excreted only ImAA-riboside. Another compound presumed to be 1,4-methylhistamine was sometimes found. Histamine-$C^{14}$ was never detected on the chromatograms, and no unusual or abnormal metabolites were discovered.[6] The absence of N-acetyl histamine was particularly significant. This substance is chromatographically identical with the so-called "conjugated histamine," which is so often measured in histamine studies of human urine (24). Although our chromatographic method is relatively insensitive, it should have detected any acetylation responsible for the large fraction of such "conjugated" histamine. Accordingly, this substance should not be used as an index of the metabolism of endogenous or parenterally administered histamine. In humans, it apparently represents mainly the action of intestinal bacteria (24).

In most of our chromatograms, ImAA was the principal urinary metabolite. Because the relative insensitivity of our chromatographic method might have led us to overlook histamine and other minor metabolites, exposure of some chromatograms to X-ray film for three months was particularly important. Significantly, this merely revealed further film reduction by radioactivity of previously identified metabolites. No new radioactive areas appeared.[6] Accordingly, our observations established with reasonable certainty that no qualitative abnormalities of histamine degradation exist in the disorders studied.

Quantitation of the radioactive metabolites was not so satisfactory. Even before development of the chromatogram, radioactive metabolites were irreversibly adsorbed by the filter paper so that only about 85 per cent of the radioactivity of a drop of a methanol extract applied to the paper could be eluted. Sensitivity of the method was also limited, due to the large amount of interfering substances which persist even after methanol extraction. Thus, only a few hundredths of a microgram of the substances of interest could be successfully chromatographed. In addition, the great variation from subject to subject (Table I) meant that any quantitative changes in disease would need to be appreciable before any reasonable inferences could be made. Detection of minor changes would require a prohibitive number of studies. Thus, although no measurable abnormalities were detected by our methods, subtle differences have not been ruled out.

The variations in metabolite distribution among individuals were noted also by Schayer and Nilsson and associates, who used isotopic dilution techniques to identify the metabolites of histamine-$C^{14}$ in human urine (6, 7). Our results do not differ qualitatively from theirs, although we found more ImAA and less meImAA in most cases. The reason for this difference is unclear, but is probably related to the method of metabolite identification rather than to the mode of histamine-$C^{14}$ administration (7).

Since injected histamine-$C^{14}$ is so rapidly metabolized and appears only in trace amounts in urine, it is questionable whether urinary assays of histamine in bound or unbound form really reflect the release of endogenous histamine. It is unlikely that urinary ImAA excretion will serve as a better index of histamine turnover, since

[6] When relatively large volumes of urine were passed through Decalso cation exchange columns, and the eluate extracted as in the chemical method for histamine determination (17). The "histamine" radioactivity thus isolated amounted to less than 5 per cent of the total radioactivity.

this substance is also derived directly from histidine catabolism (1, 25).

Precise measurements of plasma histamine levels may provide valuable information in those conditions such as asthma and anaphylaxis in which a high rate of histamine release might occur. However, our observations indicate that plasma levels would not change significantly unless a very large amount of free histamine were continually produced. Our curves of $C^{14}$ plasma histamine clearance are not precise enough for any estimation of the amount required.

Elevated blood histamine levels were found in marked hepatic insufficiency by Mitchell, Butt and Code several years ago (13). Since plasma levels were not determined, it is difficult to interpret their findings, but we could establish no deficiency in the rate of histamine metabolism.

The nature of histamine production and release in disease is certainly of primary importance, and perhaps can be studied with more confidence now that a possible variable, namely, the mode and rate of histamine metabolism, appears to be surprisingly resistant to change.

## SUMMARY

1. The metabolism of histamine-$C^{14}$ has been investigated in seven control subjects, and in five patients with Laennec's cirrhosis, three with bronchial asthma, three with histaminic cephalalgia, and one with uremia due to chronic glomerulonephritis.

2. The radioactive histamine was rapidly cleared from the blood, and excretion of radioactivity in the urine was usually complete within 30 hours.

3. Separation of the excreted radioactive metabolites by paper chromatography and radioautography showed imidazoleacetic acid to be the principal product of histamine metabolism. Imidazoleacetic acid-riboside and 1,4-methylimidazoleacetic acid were also consistently recovered in the urine, but histamine-$C^{14}$ was not detected.

4. Histamine inactivation and degradation in man is a rapid and complete process, and by our methods, no abnormalities were found in the diseases studied.

## ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of Mrs. Sue R. Stevens.

## REFERENCES

1. Schayer, R. W. Catabolism of physiological quantities of histamine *in vivo*. Physiol. Rev. 1959, **39**, 116.
2. Lindahl, K. M. On the biological methylation of histamine. Ark. Kemi 1958, **13**, 149.
3. Lindahl, K. M. On the biological methylation of histamine. Acta chem. scand. 1958, **12**, 1690.
4. Lindahl, K. M. On the biological methylation of histamine. 3. Acta chem scand. 1958, **12**, 2050.
5. Brown, D. D., Axelrod, J., and Tomchick, R. Enzymatic N-methylation of histamine. Nature (Lond.) 1959, **183**, 680.
6. Schayer, R. W., and Cooper, J. A. D. Metabolism of $C^{14}$ histamine in man. J. appl. Physiol. 1956, **9**, 481.
7. Nilsson, K., Lindell, S.-E., Schayer, R. W., and Westling, H. Metabolism of $^{14}$C-labelled histamine in pregnant and non-pregnant women. Clin. Sci. 1959, **18**, 313.
8. Adam, H. M., Hunter, R. B., and Kinnear, T. W. G. Observations on the urinary excretion of histamine in urticaria. Quart. J. exp. Physiol. 1950, **36**, 49.
9. Rose, B., Pare, J. A. P., Pump, K. K., and Stanford, R. L. Preliminary report on adrenocorticotrophic hormone (ACTH) in asthma. Canad. med. Ass. J. 1950, **62**, 6.
10. Mitchell, R. G., Logan, G. B., Peters, G. A., and Henderson, L. L. Urinary excretion of histamine in patients having asthma and hay fever: Observations on changes produced by administration of cortisone. J. Allergy 1954, **25**, 504.
11. Randolph, T. G., and Rackemann, F. M. The blood histamine level in asthma and in eosinophilia. J. Allergy 1941, **12**, 450.
12. Chambon, M., and Berthier, J. Histamine et histaminasémie au cours des lésions hépatiques. C. R. Soc. Biol. (Paris) 1945, **139**, 506.
13. Mitchell, R. G., Butt, H. R., and Code, C. F. Histamine metabolism in diseases of the liver. J. clin. Invest. 1954, **33**, 1199.
14. Mitchell, R. G. The urinary excretion of subcutaneously injected histamine. Brit. J. Pharmacol. 1956, **11**, 462.
15. Beall, G. N., and VanArsdel, P. P., Jr. Liquid scintillation counting of histamine-$C^{14}$ and its metabolites. J. Lab. clin. Med. 1959, **54**, 487.
16. Jarrett, A. A. Statistical methods used in the measurement of radioactivity. (Cited in Aronoff, S. Techniques of Radiobiochemistry. Ames, Iowa State College Press, 1956, pp. 207–214.)
17. Lowry, O. H., Graham, H. T., Harris, F. B., Priebat, M. K., Marks, A. R., and Bregman, R. U. The chemical measurement of histamine in blood plasma and cells. J. Pharmacol. exp. Ther. 1954, **112**, 116.
18. Rosiere, C. E., and Grossman, M. I. An analog of histamine that stimulates gastric acid secretion

without other actions of histamine. Science 1951, **113**, 651.

19. Ames, B. N., and Mitchell, H. K. The paper chromatography of imidazoles. J. Amer. chem. Soc. 1952, **74**, 252.
20. Karjala, S. A., and Turnquest, B. W., III. The characterization of two methylimidazoleactic acids as radioactive histamine metabolites. J. Amer. chem. Soc. 1955, **77**, 6358.
21. Curry, J. J. The action of histamine on the respiratory tract in normal and asthmatic subjects. J. clin. Invest. 1946, **25**, 785.
22. Horton, B. T. Histaminic cephalalgia. J. Amer. med. Ass. 1956, **160**, 468.
23. Tabor, H., and Hayaishi, O. The excretion of imidazoleactic acid riboside following the administration of imidazoleacetic acid or histamine to rats. J. Amer. chem. Soc. 1955, **77**, 505.
24. Urbach, K. F. Nature and probable origin of conjugated histamine excreted after ingestion of histamine. Proc. Soc. exp. biol. (N. Y.) 1949, **70**, 146.
25. Brown, D. D. The mammalian urinary metabolites of l-histidine. Fed. Proc. 1959, **18**, 198.

# ON THE MECHANISM OF IMPAIRMENT OF RENAL CONCENTRATING ABILITY IN POTASSIUM DEFICIENCY *

By ANDRZEJ MANITIUS,† HOWARD LEVITIN,‡ DAVID BECK§ AND FRANKLIN H. EPSTEIN ||

*(From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.)*

(Submitted for publication October 14, 1959; accepted December 17, 1959)

A decrease in the ability of the kidneys to excrete a highly concentrated urine is one of the earliest hallmarks of potassium depletion in animals and in humans (1). According to present views of the mechanism of the renal concentrating process (2), this might be the result of *a*) impairment of the countercurrent multiplier system by which a high concentration of sodium (and urea) is created and maintained in the interstitial space of the renal papilla, and/or *b*) decreased permeability of the walls of the collecting ducts and distal tubules to the back-diffusion of water. If the first alternative were correct, analysis of the renal papilla from potassium-deficient animals excreting a maximally concentrated urine should reveal a lower concentration of sodium than that present in the papilla of normal animals. Should the second mechanism be operative, the fall in maximum urinary osmolality observed in potassium deficiency might be entirely unassociated with a decrease in papillary sodium, or else would be out of proportion to it.

In the present experiments samples of maximally concentrated urine and renal papilla, medulla and cortex from potassium-depleted rats and dogs were analyzed. By placing all animals on a sodium-free diet prior to sacrifice, difficulties in the interpretation of tissue analyses consequent to high concentrations of sodium in the urine were avoided. The results indicate that the concentration of sodium and total solutes in the renal papilla is indeed decreased by potassium depletion, though the decrease is not as great as the fall in maximum urinary osmolality.

* Aided by grants from the American Heart Association, the National Heart Institute, the Lawrence M. Gelb Foundation, and a contract (MD-116) with the Office of the Surgeon General, Department of the Army.

† Special Fellow of the Rockefeller Foundation. Present address: Second Medical Service, Medical Academy, Gdansk, Poland.

‡ Advanced Fellow of the American Heart Association.

§ Fellow of the Dazian Foundation.

|| Established Investigator, American Heart Association.

## METHODS

### *I. Rats*

White male Sprague-Dawley rats initially weighing 150 to 200 g were divided into the following groups. The composition of the diets is given in Table I.

*Group IE.* Twenty-four animals were fed a low potassium diet for 29 days, followed by 5 days of a low sodium, low potassium diet before sacrifice.

*Group IC.* Twenty-two rats received a normal diet for 23 days, followed by 5 days of a low sodium diet.

*Group IIE.* Twenty-eight rats received a high sodium, low potassium diet for 6 days. Desoxycorticosterone acetate (DCA), 0.2 mg per rat, was injected subcutaneously daily for 5 days. Following this, a low sodium, low potassium diet was given for 9 days.

*Group IIC.* Twenty-four animals received a high sodium diet for 6 days and a low sodium diet for 5 days.

In order to obtain enough papillary tissue for accurate analysis, 3 or 4 animals were kept in a cage and analyses of urine, plasma and tissue were performed on pooled material.

Twenty-four hours before sacrifice food and water were withheld. Twelve hours later 50 mU of vasopressin in oil was injected subcutaneously and micturition was induced. The rats were then placed in metabolism cages with screens so placed as to deflect feces, and urine was collected under oil for 12 hours. Although precautions were taken to prevent evaporation, some (estimated from separate experiments as under 15 per cent) undoubtedly occurred as the urine passed over the screen and into the funnels. Rats were then removed from their cages, anesthetized with sodium pentobarbital, and the abdominal cavity was opened. Five ml of blood was aspirated anaerobically from the abdominal aorta and immediately thereafter both kidneys and about 4 g of thigh muscle were removed. Separate experiments established that the concentration of sodium in the renal papilla was not appreciably affected by preliminary bleeding in the manner described. The kidneys were sectioned longitudinally and slightly off center so that free access to the entire papilla was obtained (Figure 1A). The papilla was removed approximately at the point in-

TABLE I

*Composition of diets*

| | Normal | Low potassium | High sodium | High sodium, low potassium | Low sodium, normal potassium | Low sodium, low potassium |
|---|---|---|---|---|---|---|
| | g | g | g | g | g | g |
| NaCl | 6.04 | 6.04 | 19.52 | 19.52 | | |
| $NaH_2PO_4 \cdot H_2O$ | | 4.09 | | | | |
| KCl | | | | | 7.4 | |
| $K_2HPO_4$ | 11.60 | | 11.60 | | 2.4 | |
| Mineral mixture | 30.37 | 30.37 | 30.37 | 30.37 | 30.37 | 30.37 |
| Basic diet | ad 1,000.0 | ad 1,000.0 | ad 1,000.0 | ad 1,000.0 | ad 1,000.0 | ad 1,000.0 |
| Diet content | *mEq/kg* | *mEq/kg* | *mEq/kg* | *mEq/kg* | *mEq/kg* | |
| Na | 103 | 133 | 334 | 334 | | |
| K | 133 | | 133 | | 114 | |
| Cl | 103 | 103 | 334 | 334 | 100 | |

Mineral mixture

| | g |
|---|---|
| $CaCO_3$ | 2,400 |
| $CaHPO_4$ | 496 |
| $MgSO_4$ | 1,186 |
| $MmSO_4$ | 30 |
| $ZnCl_2$ | 2.0 |
| $CuSO_4$ | 2.4 |
| KI | 6.4 |
| $CoCl_2$ | 2.4 |
| Fe-citrate | 220 |

Basic diet

| | g |
|---|---|
| Sucrose | 458 |
| Lard | 220 |
| Vitaminized casein | 250 |
| Vitaminized corn oil | 50 |
| Choline | 4 |

dicated in Figure IB. Portions of the inner medulla and cortex were then obtained.

*II. Dogs*

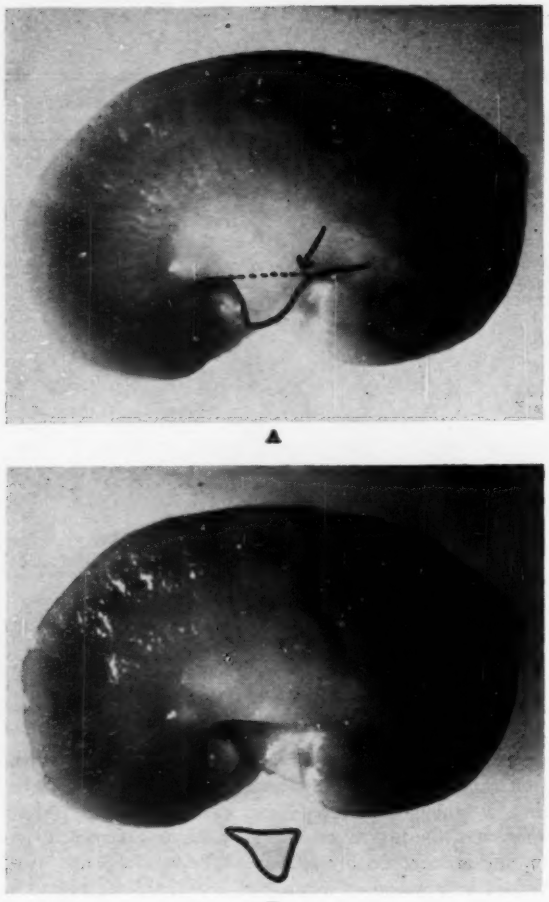
Five female mongrel dogs weighing 7 to 15 kg, were depleted of potassium by a high sodium, low potassium diet (Table I) plus 1.0 mg of DCA per kg of body weight injected subcutaneously each day for 7 to 9 days, followed by a low sodium, low potassium diet for 8 more days. A sixth animal received 45 g of potassium-exchange resin (Kayexalate, Winthrop) in its low potassium diet each day for 13 days, and was then placed on a low sodium, low potassium diet for 8 more days. Six other dogs served as controls and were fed for 4 to 8 days on the high sodium diet and then for 8 or 9 days with the low sodium diet. Water was withheld for 36 hours before sacrifice. Twelve hours and 2 hours before sacrifice, 5 units of vasopressin in oil was injected subcutaneously. A sample of venous blood was collected anaerobically and the dog was lightly anesthetized with sodium pentobarbital. (The anesthetic dose of pentobarbital is considerably reduced in potassium-depleted animals.) The bladder was emptied, washed with air, and urine was collected via an inlying catheter for the next 15 to 30 minutes. The abdomen was then opened and both kidneys were removed and sectioned transversely. Samples of papilla tip (20 to 60 mg), inner medulla (300 to 400 mg) and cortex (about 1,500 mg) were immediately removed and placed in weighing bottles.

*III. Analytical procedures*

In the rat experiments, tissue from one kidney was analyzed for urea and ammonia; tissue from the other kidney, for water and electrolytes. In the experiments on dogs, samples of each renal papilla, medulla and cortex were divided into two approximately equal portions. The portion for urea and ammonia analysis was placed in a weighing bottle containing clean dry sand. After weighing, the tissue and sand were transferred to a mortar and thoroughly ground. The mixture was suspended in a measured amount of water, transferred to a closed test tube and immersed in boiling water for 5 minutes. After cooling, the fluid was filtered and analyzed for urea and ammonia (3). The second portion of renal tissue was weighed, then desiccated for 5 days at 95° C and reweighed. Water content was determined after grinding, drying for another 24 hours at 95° C and weighing again. The ground dry tissue was then digested for 24 hours in concentrated nitric acid at 70° C. Sodium and potassium were determined by indirect flame photometry on the filtered, diluted digest.

Muscle tissue was dried, extracted three times with anhydrous ether, ground, and digested with nitric acid before determination of sodium and potassium with a flame photometer.

Osmolality of urine and plasma was measured with the Fiske osmometer. Urine was diluted to an osmolality less than 1,000 mOsm per kg prior to the determination of freezing point. $CO_2$ content of plasma was measured with the van Slyke apparatus or the Natelson microgasometer. "Total solute" concentration in tissue was calculated as the sum of $2 \times (Na + K + NH_4)$ + urea, where Na, K, $NH_4$ and urea are expressed as millimoles per kilogram of tissue water. It must be emphasized that for many reasons (4) this value is at best



A

B

FIG. 1. A. RAT KIDNEY SECTIONED LONGITUDINALLY. The arrow indicates the approximate point at which the papilla tip was excised. B. RAT KIDNEY AFTER EXCISION OF PAPILLA.

only a rough approximation of the osmotic activity of water in tissue.

## RESULTS

*1. Rats (Tables II and III).* As reported many times by others (5, 6), potassium depletion produced by dietary means alone (Group IE) and by DCA (Group IIE) was associated with decreased weight gain, polyuria, renal hypertrophy, hypokalemia, alkalosis, a decrease in muscle potassium and an increase in muscle sodium (Table II). The potassium content of the papilla and cortex of depleted rats was lower than that of controls (Table III) but this was not true for the renal medulla.

On a sodium-free diet, potassium-depleted rats

TABLE II

*Effect of potassium depletion on composition of plasma, urine and muscle of rats* *

| Groups/ animals | Weight | Plasma | | | | $U_{osm}/P_{osm}$ | Urine | | | | | | | Muscle | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Na | K | $CO_2$ | Urea | | $U_{osm}$ | Na | K | $NH_4$ | Urea | $U_{osm}V$ | $U_{Na}V$ | $H_2O$ content | Na | K |
| | *g* | *mEq/L* | *mEq/L* | *mmole/L* | *mmole/L* | | *mOsm/kg* | *mEq/L* | *mEq/L* | *mEq/L* | *mmole/L* | *mOsm/ 24 hr/ 100 g* | *μEq/ 24 hr/ 100 g* | *%* | *mEq/ 100 g FFDS†* | *mEq/ 100 g FFDS* |
| IC (control) | 6/22 | 152.0 ±2.2 | 4.57 ±0.47 | 24.8 ±1.4 | 5.24 ±0.39 | 10.10 ±0.99 | 3,132 ±290 | 23.1 ±10.9 | 530.3 ±50.6 | 219.2 ±45.5 | 1,598 ±180 | 2.24 ±0.38 | 16 ± 8 | 74.5 ±0.7 | 8.13 ±0.59 | 44.27 ±0.51 |
| IE (low K) | 7/24 | 154.4 ±1.8 | 2.12 ±0.27 | 28.6 ±1.3 | 7.42 ±0.52 | 5.60 ±1.10 | 1,749 ±326 | 36.2 ±16.4 | 5.9 ± 1.6 | 307.7 ±76.4 | 1,086 ±156 | 2.59 ±0.32 | 56 ±27 | 74.2 ±0.4 | 15.54 ±1.30 | 33.95 ±2.28 |
| p | | < 0.01 | >0.05 | <0.01 | <0.01 | <0.01 | < 0.01 | > 0.1 | < 0.01 | < 0.05 | < 0.01 | >0.5 | < 0.01 | >0.3 | <0.01 | <0.01 |
| IIC (control) | 6/24 | 148.4 ±1.0 | 3.80 ±0.31 | 23.7 ±2.35 | 6.57 ±0.39 | 9.60 ±1.21 | 2,995 ±121 | 3.4 ± 1.1 | 398.1 ±62.4 | 196.2 ±26.5 | 1,724 ±202 | 3.65 ±0.87 | 4 ± 1 | 74.8 ±0.5 | 10.05 ±1.46 | 46.52 ±0.64 |
| IIE (low K) | 7/28 | 151.7 ±2.8 | 3.17 ±0.24 | 26.5 ±4.14 | 9.44 ±1.81 | 5.27 ±1.56 | 1,673 ±478 | 34.4 ±16.4 | 8.7 ± 2.9 | 302.6 ±97.9 | 1,112 ±337 | 2.78 ±0.42 | 60 ±36 | 74.8 ±0.3 | 14.27 ±0.59 | 39.03 ±1.53 |
| p | | < 0.01 | <0.01 | >0.1 | <0.01 | <0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | <0.05 | < 0.01 | >0.9 | <0.01 | <0.01 |

* Values given are for mean and standard deviation.
† FFDS = fat-free dry solids.

TABLE III

*Effect of potassium depletion on composition of renal papilla, medulla and cortex in rats* *

| | Papilla | | | | | | | Medulla | | | | | | Cortex | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Weight | Water content | Na | K | $NH_4$ | Urea | "Total solutes"† | Na | Water content | Na | K | $NH_4$ | Urea | Water content | Na | K | $NH_4$ | Urea |
| | *mg* | % | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mmole/kg $H_2O$* | *mmole/kg $H_2O$* | *mEq/100 g DS‡* | % | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mmole/kg $H_2O$* | % | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mmole/kg $H_2O$* |
| IC (Control) | 8.2 ±2.1 | 82.6 ±1.3 | 222 ±22.0 | 83 ± 9.9 | 41 ±15.3 | 701 ±147.0 | 1,391 ±165.5 | 106 ± 7.7 | 82.9 ±1.1 | 128 ±14.5 | 79 ±4.8 | 28 ±4.6 | 292 ±65.4 | 75.6 ±0.8 | 64 ±2.4 | 103 ±5.0 | 20 ±4.3 | 33 ±6.2 |
| IE (Low K) | 10.7 ±1.9 | 80.7 ±1.4 | 187 ±42.0 | 61 ± 3.6 | 49 ± 7.1 | 404 ±141.4 | 997 ±194.3 | 77 ±13.7 | 80.7 ±1.3 | 115 ±17.4 | 78 ±2.9 | 38 ±7.3 | 131 ±57.2 | 76.7 ±0.7 | 62 ±3.0 | 91 ±4.8 | 24 ±2.1 | 29 ±5.0 |
| p | <0.01 | <0.05 | > 0.05 | < 0.01 | > 0.2 | < 0.05 | < 0.01 | < 0.01 | <0.01 | > 0.01 | >0.6 | >0.5 | < 0.01 | <0.05 | >0.2 | <0.01 | >0.2 | >0.2 |
| IIC (Control) | 6.2 ±0.6 | 82.6 ±1.5 | 242 ±34.2 | 86 ±11.8 | 137 ±33.4 | 569 ±109.9 | 1,498 ±228.7 | 115 ±11.9 | 84.2 ±1.1 | 145 ±16.5 | 74 ±4.1 | 39 ±5.1 | 317 ±59.7 | | | | | |
| IIE (Low K) | 6.7 ±1.1 | 78.8 ±0.9 | 190 ±11.5 | 68 ± 5.9 | 118 ±49.0 | 468 ±116.6 | 1,220 ±149.0 | 71 ± 4.9 | 81.4 ±0.4 | 114 ± 9.3 | 78 ±1.6 | 37 ±6.1 | 171 ±17.1 | | | | | |
| p | >0.1 | <0.01 | < 0.01 | < 0.01 | > 0.2 | > 0.1 | < 0.05 | < 0.01 | <0.01 | < 0.01 | <0.05 | >0.5 | < 0.01 | | | | | |

* Values given are for mean and standard deviation.
† "Total solutes" = $2 \times (Na + K + NH_4) + urea$.
‡ DS = dry solids.

excreted more sodium in the urine than did normal controls deprived of sodium for a similar or shorter period (Table II).

The concentration of sodium and urea in renal tissue of normal rats increased progressively from cortex to papilla (Table III), as was reported by Ullrich and Jarausch in dogs (7). No such gradient was observed for potassium. The concentration of sodium in water of papillary tissue was considerably higher than that in peripheral plasma, even though the sodium concentration of urine was extremely low. "Total solute" concentration of papillary tissue was about one-half the osmolality of urine, but tended to increase as the sample excised for analysis became smaller and smaller as the tip of the papilla was approached, (Figure 2). The concentration of potassium in the urine of normal rats on a sodium-free diet was so high (400 to 500 mEq per L) when compared with the concentration of potassium in the renal papilla (70 to 90 mEq per kg $H_2O$) that urine could not possibly have made up more than 15 to 20 per cent of the water of the papilla, and probably was a good deal less than that.

Maximum urinary osmolality was approximately halved by potassium depletion. This was associated in both groups of depleted rats with a significant reduction in the amount of sodium per 100 g of dry solids of renal papilla. In group IE, the decrease in concentration of sodium, expressed as milliequivalents per kilogram of tissue

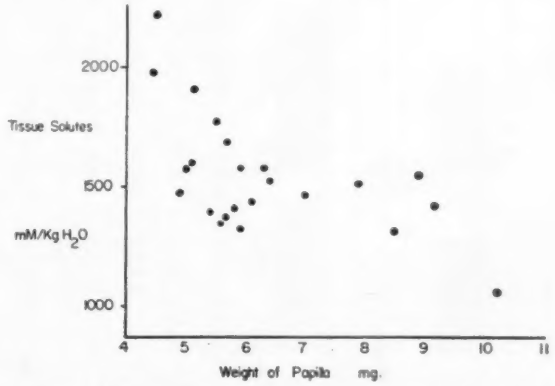



FIG. 2. CONCENTRATION OF "TISSUE SOLUTES" [$2 \times (Na + K + NH_4) + UREA$] IS PLOTTED VERSUS WEIGHT OF ANALYZED SAMPLE OF PAPILLA. As the tip of the papilla is approached (i.e., as the sample analyzed becomes smaller) the concentration of solutes in tissue water rises.

TABLE IV

*Effect of potassium depletion on composition of urine and plasma in hydropenic dogs* *

| | Animals | Weight | Plasma | | | | Urine | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Na | K | $CO_2$ | Urea | $\frac{U_{osm}}{P_{osm}}$ | $U_{osm}$ | Na | K | $NH_4$ | Urea | $U_{osm}V$ | $U_{Na}V$ |
| | *no.* | *kg* | *mEq/L* | *mEq/L* | *mmole/L* | *mmole/L* | | *mOsm/kg* | *mEq/L* | *mEq/L* | *mEq/L* | *mmole/L* | *μOsm/min/kg body wt* | *μEq/min/kg body wt* |
| Normal | 6 | 7.9–14.8 | 154.3 | 4.28 | 22.5 | 7.91 | 6.31 | 1,966 | 36.8 | 257.7 | 82.7 | 1,254 | 18 | 0.37 |
| | | | ±4.0 | ±0.45 | ±1.8 | ±1.81 | +1.18 | ±388 | ±29.1 | ±115.6 | ±38.7 | ±145 | ±8 | ±0.33 |
| Low K | 6 | 7.9–14.3 | 156.8 | 3.58 | 23.5 | 9.46 | 2.91 | 910 | 100.5 | 23.0 | 42.2 | 532 | 20 | 2.23 |
| | | | ±2.9 | ±0.34 | ±2.6 | ±2.13 | ±0.94 | ±298 | ±54.5 | ± 17.6 | ±27.2 | ±132 | ±9 | ±1.30 |
| p | | | >0.2 | >0.3 | >0.4 | >0.2 | <0.01 | < 0.01 | < 0.02 | < 0.01 | > 0.05 | < 0.01 | >0.6 | <0.01 |

* Values are for mean and standard deviation.

TABLE V

*Effect of potassium depletion on composition of renal papilla, medulla and cortex in hydropenic dogs* *

| | | Papilla | | | | | | | Medulla | | | | | Cortex | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Weight | Water content | Na | K | $NH_4$ | Urea | "Tissue solutes"† | Na | Water content | Na | K | $NH_4$ | Urea | Water content | Na | K | $NH_4$ | Urea |
| | *mg* | % | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mmole/kg $H_2O$* | *mmole/kg $H_2O$* | *mEq/100 g DS‡* | % | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mmole/kg $H_2O$* | % | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mEq/kg $H_2O$* | *mmole/kg $H_2O$* |
| Normal | 36.9 | 83.3 | 309 | 72 | 93 | 675 | 1,620 | 151.8 | 84.2 | 213 | 47 | 19 | 489 | 80.0 | 66 | 72 | 10 | 34 |
| | | ±2.3 | ±83.6 | ±10.0 | ±63.3 | ±316.2 | ±374.9 | ±21.4 | ±2.0 | ±52.2 | ±1.0 | ±5.9 | ±157.4 | ±0.6 | ±9.4 | ±4.2 | ±2.2 | ±10.4 |
| Low K | 41.3 | 84.5 | 198 | 56 | 47 | 390 | 991 | 106.5 | 84.5 | 167 | 44 | 12 | 288 | 81.5 | 71 | 62 | 11 | 44 |
| | | ±0.4 | ±57.4 | ± 7.4 | ±24.7 | ±127.5 | ±208.0 | ±22.5 | ±1.9 | ±71.0 | ±7.9 | ±3.9 | ±109.7 | ±1.4 | ±9.0 | ±9.4 | ±1.8 | ±34.8 |
| p | | >0.2 | < 0.05 | < 0.02 | > 0.1 | < 0.01 | < 0.01 | < 0.01 | >0.7 | > 0.2 | >0.3 | <0.05 | < 0.05 | <0.05 | >0.3 | <0.05 | >0.4 | > 0.5 |

* Values given are for mean and standard deviation.
† "Tissue solutes" = 2 × (Na + K + $NH_4$) + urea.
‡ DS = dry solids.

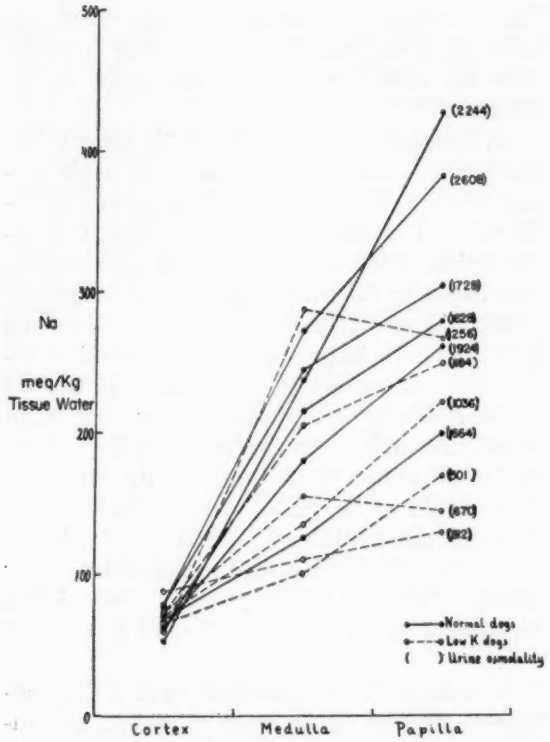



FIG. 3. GRADIENT OF SODIUM CONCENTRATION FROM CORTEX TO MEDULLA IS PLOTTED INDIVIDUALLY FOR NORMAL DOGS (SOLID LINES) AND POTASSIUM-DEPLETED DOGS (BROKEN LINES). The concentration of sodium in the papilla is reduced in potassium-deficient animals, although not so much as is the maximal urinary osmolality (in parentheses).

water, did not reach statistical significance, but in group IIE a significant decrease in the concentration of sodium in tissue water was apparent in both medulla and papilla. An unexpected decrease in the water content of papilla and medulla was apparent in both groups of potassium-deficient rats. "Total solute" concentration of papilla and medulla was significantly diminished by potassium deficiency, as a result of decreases in the concentration of both sodium and urea.

*II. Dogs (Tables IV and V).* Potassium-depleted dogs became weak, and some (not included here) died with symptoms of paralysis of peripheral and respiratory muscles. Urinary excretion of potassium was extremely low and, as in potassium-depleted rats, difficulty in conserving sodium was noted on a sodium-free diet.

Potassium deficiency was associated with a

TABLE VI

*Comparison of urinary osmolality, papillary solute concentration and papillary sodium concentration in normal and potassium-depleted dogs*

| | Animal | $U_{osm}$ | "Tissue solutes"* | Papillary Na |
|---|---|---|---|---|
| | *no.* | *mOsm/kg $H_2O$* | *mmole/kg $H_2O$* | *mEq/kg $H_2O$* |
| Normal dogs | 75 | 1,628 | 1,448 | 279 |
| | 74 | 1,664 | 1,232 | 198 |
| | 69 | 1,728 | 1,518 | 304 |
| | 73 | 1,924 | 1,339 | 262 |
| | 67 | 2,244 | 2,006 | 428 |
| | 57 | 2,608 | 2,181 | 382 |
| Potassium-depleted dogs | 65 | 501 | 772 | 172 |
| | 63 | 670 | 672 | 146 |
| | 58 | 812 | 916 | 129 |
| | 53 | 1,036 | 1,076 | 222 |
| | 70 | 1,184 | 1,174 | 250 |
| | 61 | 1,256 | 1,336 | 269 |

* "Tissue solutes" = $2 \times (Na + K + NH_4)$ + urea.

marked decrease in maximal urinary osmolality and with a significant fall in the concentration of sodium and urea in the renal papilla. (The decrease in water content of medulla and papilla observed in potassium-deficient rats was not noted in dogs.) Figure 3 illustrates the decreased gradient of sodium concentration between cortex, medulla and papilla produced in dogs by potassium depletion.

The calculated solute concentration of the papilla of normal dogs was about 80 per cent of the

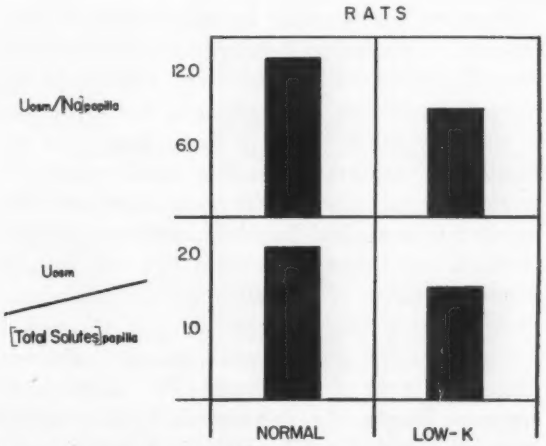



FIG. 4. POTASSIUM DEPLETION PRODUCED A DECREASE IN RATIO OF MAXIMAL URINARY OSMOLALITY ($U_{OSM}$) TO CONCENTRATION (IN MILLIMOLES PER KILOGRAM TISSUE WATER) OF SODIUM AND OF "TOTAL SOLUTES" IN RENAL PAPILLA.

osmolality of urine. In potassium-deficient dogs, on the other hand, total solute concentration of the papilla was equal to or higher than the osmolality of urine (Table VI). These data suggest but do not prove the possibility that the back-diffusion of water from collecting ducts into the interstitium of the papilla may have been impaired by potassium deficiency. Similar alterations were observed in the ratio of urinary osmolality to the concentration of total solutes or sodium in the papilla of potassium-deficient rats (Figure 4), although in the rat experiments urine from several rats was pooled and collected in funnels for 12 hours prior to sacrifice, and the absolute values of such ratios are therefore less meaningful.

## DISCUSSION

These experiments illustrate clearly the effect of potassium depletion in impairing the ability of the kidneys to produce a concentrated urine. Although desoxycorticosterone was used in some rats and dogs to induce potassium deficiency, the results cannot be ascribed to the influence of this hormone, per se, on the renal tubules, since its administration was discontinued many days before the animals were sacrificed. It is unlikely that the low sodium diet to which all animals were subjected substantially affected maximum urinary concentration or the composition of the renal papilla (8).

*A priori*, the decrease in concentrating ability observed in potassium deficiency might arise from several sources. First, the distal convoluted tubules and collecting ducts might be less permeable to the outward diffusion of water under the influence of vasopressin, so that water would be retained in the urine despite the maintenance of a hypertonic interstitial fluid in medulla and papilla. Giebisch and Lozano concluded that this was an important cause of hyposthenuria in potassium-depleted dogs, because these animals excreted a hypotonic urine during mild mannitol diuresis despite infusions of vasopressin (9). In the present experiments, the decrease in urinary solute concentration induced by potassium depletion always exceeded the fall in the concentration of sodium or total solutes in the water of the papilla, and presumably, in the papillary interstitium. These findings are compatible with some impairment of the back-diffusion of water from the collecting ducts, but they do not suffice to establish it.

An increased delivery of hypotonic urine to the distal convolutions and collecting ducts might also play a role in the hyposthenuria of potassium deficiency. It is conceivable, for example, that reabsorption of salt and water in the proximal tubule might be diminished, and that consequently more fluid would be delivered to the ascending limb of Henle's loop, where sodium would be reabsorbed without water. The present data do not bear directly on this possibility, but one might expect that under such circumstances the concentration of sodium in the tissue of the renal medulla would be increased over normal or unchanged, not diminished. It is noteworthy in this connection that the total solute excretion per minute (corrected for body weight) was similar for potassium-depleted animals and for normal controls.

A decrease in the concentration of sodium attained in the interstitial fluids of medulla and papilla would be expected to result in hyposthenuria. The present experiments suggest that this is an important cause of the decrease in renal concentrating ability associated with potassium deficiency. The diminished concentration of sodium in papillary *tissue* cannot be ascribed to a lower concentration of sodium in the *urine* of the potassium-depleted animals, since urinary sodium concentration in potassium deficiency was actually higher than that of control animals. Although an increased *volume* of sodium-poor urine in the collecting ducts of potassium-deficient, polyuric animals might have reduced the papillary concentration of sodium expressed as milliequivalents per kilogram of tissue water, it would not have produced a decrease in the amount of sodium per 100 g of dry solids.

The decrease in sodium concentration in renal medulla and papilla observed in potassium-deficient animals might result from *1*) alterations in countercurrent flow through capillary loops of the vasa recta, *2*) impairment of sodium reabsorption from the loops of Henle, or *3*) impairment of active reabsorption of sodium without water from the collecting ducts. The data do not

bear critically on the first possibility, which is difficult to test with present methods. With regard to the second and third alternatives, the tendency of potassium-depleted rats and dogs on a sodium-free diet to lose sodium in the urine suggests impairment of tubular reabsorption of sodium at some site in the nephron. Some sodium transport mechanisms are known to be sensitive to lack of potassium (10, 11). The high concentration of sodium normally present in the renal medulla is thought to be a result of active transport of sodium by the loops of Henle (12). Nevertheless, several considerations suggest that in potassium deficiency, impaired reabsorption of sodium from the collecting ducts may be in part responsible for the diminished accumulation of sodium in the papilla. The osmolality of fluid obtained by micropuncture from the early distal convolution of hydropenic potassium-depleted rats is hypotonic to plasma and similar to that of control animals (13). Potassium-deficient rats with impaired *concentrating* ability are able to *dilute* the urine normally in response to a water load (14). These facts suggest that in potassium deficiency reabsorption of sodium without water proceeds normally in the loop of Henle. Finally, the alterations in histological structure of the rat kidney produced by potassium depletion are most marked in the collecting ducts (15).

Active reabsorption of sodium from the collecting ducts has been demonstrated by Hilger, Klümper and Ullrich (16). As pointed out by these authors and by Gottschalk and Mylle (12), if during hydropenia the collecting ducts are freely permeable to water, the mere removal of sodium from the urine by collecting duct epithelium might serve to *maintain* the hypertonicity of medullary interstitial fluid but could not *increase* it. If, however, sodium reabsorption in this region were linked primarily or partly with the creation of osmotically active particles (for example, the addition to the urine of $NH_4^+$) (17), sodium reabsorbed by the collecting ducts could be trapped in the medulla by countercurrent capillary flow (18) and could contribute importantly to the creation of an osmotic gradient between cortex and medulla. It seems reasonable to suppose that the collecting ducts play a role in normal kidneys in establishing this gradient and that this process is disrupted in potassium deficiency.

## SUMMARY

1. The effect of potassium depletion on the composition of renal papilla, medulla and cortex was studied in dogs and rats in an effort to clarify the mechanism of the hyposthenuria produced by potassium deficiency.

2. Potassium deficiency was associated with a fall in the concentration of sodium and urea in the papilla and medulla of hydropenic animals.

3. The ratio of urinary solute concentration to papillary solute concentration was uniformly lower in potassium-depleted than in normal animals. This finding is compatible with but does not prove the hypothesis that potassium deficiency impairs the permeability of the collecting ducts to water.

4. It is suggested that the defect in renal concentrating ability induced by potassium deficiency may be at least in part a result of impaired reabsorption of sodium by the collecting ducts.

## ACKNOWLEDGMENT

The authors acknowledge gratefully the technical assistance of Mrs. Nadia Myketey and Mrs. Eva Taborsky.

## REFERENCES

1. Relman, A. S., and Schwartz, W. B. The kidney in potassium depletion. Amer. J. Med. 1958, **24**, 764.
2. Smith, H. W. The fate of sodium and water in the renal tubules. Bull. N. Y. Acad. Med. 1959, **35**, 293.
3. Conway, E. J. Microdiffusion Analysis and Volumetric Error, 4th ed. London, Crosby Lockwood and Son, Ltd., 1957.
4. Maffly, R. H., and Leaf, A. The potential of water in mammalian tissues. J. gen. Physiol. 1959, **42**, 1257.
5. Hollander, W., Jr., Winters, R. W., Williams, T. F., Bradley, J., Oliver, J., and Welt, L. G. Defect in the renal tubular reabsorption of water associated with potassium depletion in rats. Amer. J. Physiol. 1957, **189**, 557.
6. Muntwyler, E., and Griffin, G. E. Tissue electrolyte content of potassium and protein-deficient rats. Proc. Soc. exp. Biol. (N. Y.) 1955, **89**, 349.
7. Ullrich, K. J., and Jarausch, K. H. Untersuchungen zum Problem der Harnkonzentrierung und Harnverdünnung. Pflüg. Arch. ges. Physiol. 1956, **262**, 537.

8. Manitius, A., Levitin, H., Beck, D., and Epstein, F. H. Unpublished observations.
9. Giebisch, G., and Lozano, R. The effects of adrenal steroids and potassium depletion on the elaboration of an osmotically concentrated urine. J. clin. Invest. 1959, **38**, 843.
10. Huf, E. G., and Wills, J. Influence of some inorganic cations on active salt and water uptake by isolated frog skin. Amer. J. Physiol. 1951, **167**, 255.
11. Ussing, H. H. Ion transport across biological membranes *in* Ion Transport across Membranes. New York, Academic Press Inc., 1959.
12. Gottschalk, C. W., and Mylle, M. Micropuncture study of the mammalian urinary concentrating mechanism: Evidence for the countercurrent hypothesis. Amer. J. Physiol. 1959, **196**, 927.
13. Gottschalk, C. W., Mylle, M., Winters, R. W., and Welt, L. G. Micropuncture study of the osmolality of renal tubular fluid in potassium-depleted rats (abstract). J. clin. Invest. 1958, **37**, 898.
14. Levitin, H., Manitius, A., Beck, D., and Epstein, F. H. Urinary dilution in potassium deficiency. To be published.
15. Oliver, J., MacDowell, M., Welt, L. G., Holliday, M. A., Hollander, W., Jr., Winters, R. W., Williams, T. F., and Segar, W. E. The renal lesions of electrolyte imbalance. I. The structural alterations in potassium-depleted rats. J. exp. Med. 1957, **106**, 563.
16. Hilger, H. H., Klümper, J. D., and Ullrich, K. J. Wasserrückresorption und Ionentransport durch die Sammelrohrzellen der Säugetierniere. Pflüg. Arch. ges. Physiol. 1958, **267**, 218.
17. Ullrich, K. J., Hilger, H. H., and Klümper, J. D. Sekretion von Ammoniumionen in den Sammelrohren der Säugetierniere. Pflüg. Arch. ges. Physiol. 1958, **267**, 244.
18. Berliner, R. W., Levinsky, N. G., Davidson, D. G., and Eden, M. Dilution and concentration of the urine and the action of antidiuretic hormone. Amer. J. Med. 1958, **24**, 730.

# ON THE MECHANISM OF IMPAIRMENT OF RENAL CONCENTRATING ABILITY IN HYPERCALCEMIA *

By ANDRZEJ MANITIUS,† HOWARD LEVITIN,‡ DAVID BECK§ AND FRANKLIN H. EPSTEIN ||

*(From the Department of Internal Medicine, Yale University School of Medicine, New Haven, Conn.)*

(Submitted for publication October 14, 1959; accepted December 17, 1959)

A decrease in the ability of the kidneys to concentrate the urine is characteristic of many varieties of experimental and clinical hypercalcemia, hypercalciuria and nephrocalcinosis (1). Vitamin D intoxication in rats (2) and large doses of parathyroid extract in dogs (3, 4), in addition to producing hyposthenuria, have been shown to cause morphological changes which are most marked in the epithelial cells lining the collecting ducts but are also present in the distal convoluted tubules and the loops of Henle. Such lesions might impair renal concentrating capacity by interfering with the accumulation and concentration of sodium in the interstitial fluids of the medulla and papilla, or by impairing the back-diffusion of water from the lumen of the collecting duct into a hypertonic interstitium (5).

In the present studies the composition of renal cortex, medulla and papilla was compared with that of plasma and urine in hydropenic rats in which hypercalcemia had been induced by large doses of vitamin D. The results indicate that the hyposthenuria observed in such animals results at least in part from a diminished concentration of sodium in renal medulla and papilla.

## MATERIALS AND METHODS

White male Sprague-Dawley rats weighing 150 to 200 g were fed a synthetic diet containing liberal amounts of sodium and potassium (6). Forty-five animals divided into 12 groups were given subcutaneously, daily for 4 days, 400,000 units of vitamin $D_2$ suspended in peanut oil; following this they were placed on a diet containing no sodium (6) for 5 to 10 days. Seven to 12 days after the last injection of calciferol, the animals were sacrificed and portions of renal papilla, medulla and cortex were obtained for analysis. Seventy rats divided into 18 groups served as controls, receiving a diet free of sodium for 5 days before sacrifice. In order to obtain enough tissue for accurate analysis, 3 or 4 rats were kept in a single cage and the material obtained from all animals in a group was pooled. Prior to sacrifice urine was collected for 12 hours under the influence of hydropenia and exogenous vasopressin. The techniques of collection and analysis of blood, urine and tissue have been described (6).

## RESULTS

Rats given calciferol appeared lethargic and weak. Polyuria was apparent and the level of urea nitrogen in plasma was mildly elevated (Table I). At the time of sacrifice, serum calcium had risen to an average of 15.7 mg per 100 ml.

As previously reported (2), maximum urinary osmolality was greatly diminished in rats injected with vitamin D (control = 3,036 mOsm per kg; vitamin D = 1,724 mOsm per kg). This could not be ascribed to a solute diuresis, since the total solute output of the hypercalcemic animals did not differ significantly from that of controls. Despite the fact that vitamin D-intoxicated rats were kept on a sodium-free diet as long or longer than were the controls, their losses of sodium in the urine were significantly greater than in the normal group.

The sodium content of papilla and medulla of hypercalcemic rats was lower than that of normal animals (Table II). This difference was highly significant, whether expressed as milliequivalents per kilogram of tissue water or as milliequivalents per 100 g of dry solids. The concentration of urea in papilla and medulla was likewise depressed, so

* Aided by grants from the American Heart Association, the National Heart Institute, the Lawrence M. Gelb Foundation, and a contract (MD-116) with the Office of the Surgeon General, Department of the Army.

† Special Fellow of the Rockefeller Foundation. Present address: Second Medical Service, Medical Academy, Gdansk, Poland.

‡ Advanced Fellow of the American Heart Association.

§ Fellow of the Dazian Foundation.

|| Established Investigator, American Heart Association.

TABLE I

*Effect of nephrocalcinosis on plasma and urine of rats* *

| | | | Plasma | | | | | | Urine | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Groups/ animals | Weight | Osmol-ality | Na | K | $CO_2$ | Urea | Ca | Osmolal U/P ratio | $U_{osm}$ | Na | K | $NH_4$ | Urea | Ca | $U_{osm}V$ | $U_{Na}V$ |
| | | *g* | *mOsm/kg* | *mEq/L* | *mEq/L* | *mmole/L* | *mmole/L* | *mg/ 100 ml* | | *mOsm/kg* | *mEq/L* | *mEq/L* | *mEq/L* | *mmole/L* | *mg/ 100 ml* | *mOsm/ 24 hr/ 100 g* | *μEq/ 24 hr/ 100 g* |
| Control | 18/70 | 250 | 313 | 150.0 | 4.17 | 24.3 | 6.6 | 10.2 | 9.70 | 3,036 | 15.2 | 395.1 | 226.0 | 1,719 | 8.1 | 3.57 | 17 |
| SD | | ±60 | ±5 | ±2.3 | ±0.50 | ± 1.8 | ± 1.4 | ±0.3 | ±1.00 | ±184 | ±13.3 | ±108.8 | ±44.4 | ±200 | ± 2.2 | ±1.28 | ± 8 |
| Vit. D | 12/45 | 237 | 325 | 150.3 | 3.98 | 25.0 | 14.7 | 15.7 | 5.30 | 1,724 | 41.8 | 205.7 | 98.7 | 969 | 89.4 | 4.13 | 100 |
| SD | | ±45 | ±5 | ±1.6 | ±0.27 | ± 1.2 | ± 3.9 | ±0.6 | ±0.74 | ±261 | ±17.2 | ± 38.8 | ±41.2 | ±134 | ±39.3 | ±0.80 | ±43 |
| p | | > 0.5 | <0.01 | >0.7 | >0.2 | > 0.2 | <0.01 | <0.01 | <0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | < 0.01 | >0.1 | <0.01 |

* Values are given for mean and standard deviation.

TABLE II

*Effect of nephrocalcinosis on composition of renal tissue* *

| | | Papilla | | | | | | | Medulla | | | | | | Cortex† | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Weight | Water content | Na | Na | K | $NH_4$ | Urea | Total solutes‡ | Water content | Na | Na | K | $NH_4$ | Urea | Water content | Na | K | $NH_4$ | Urea |
| | *mg* | % | *mEq/ kg $H_2O$* | *mEq/ 100 g DS§* | *mEq/ kg $H_2O$* | *mEq/ kg $H_2O$* | *mmole/ kg $H_2O$* | *mmole/ kg $H_2O$* | % | *mEq/ kg $H_2O$* | *mEq/ 100 g DS* | *mEq/kg $H_2O$* | *mEq/ kg $H_2O$* | *mmole/ kg $H_2O$* | % | *mEq/ kg $H_2O$* | *mEq/ kg $H_2O$* | *mEq/ kg $H_2O$* | *mmole/ kg $H_2O$* |
| Control | 6.6 | 82.6 | 241 | 114 | 83 | 101 | 598 | 1,460 | 83.7 | 141 | 73 | 75 | 33 | 302 | 75.6 | 64 | 103 | 20 | 33 |
| SD | ±1.3 | ±1.3 | ±32 | ±12 | ±10 | ±54 | ±142 | ±201 | ±1.1 | ±17 | ±11 | ±4 | ±7 | ±66 | ±0.8 | ±2 | ±5 | ±4 | ±6 |
| Vit. D | 7.2 | 84.5 | 178 | 96 | 84 | 50 | 374 | 995 | 85.0 | 108 | 62 | 77 | 26 | 212 | 76.5 | 67 | 103 | 16 | 40 |
| SD | ±2.7 | ±1.0 | ±36 | ±17 | ±10 | ±18 | ±57 | ±121 | ±1.3 | ± 8 | ± 5.5 | ±5 | ±6 | ±31 | ±0.8 | ±4 | ±7 | ±2 | ±9 |
| p | NS‖ | <0.01 | < 0.01 | < 0.01 | NS | < 0.01 | < 0.01 | < 0.01 | <0.01 | < 0.01 | < 0.01 | NS | <0.01 | < 0.01 | NS | NS | NS | NS | NS |

* Values are given for mean and standard deviation.
† Cortices of only 6 control and 6 vitamin D groups were analyzed.
‡ "Total solutes" = 2 (Na + K + $NH_4$) + urea.
§ DS = dry solids.
‖ NS = not significant ($p > 0.05$).

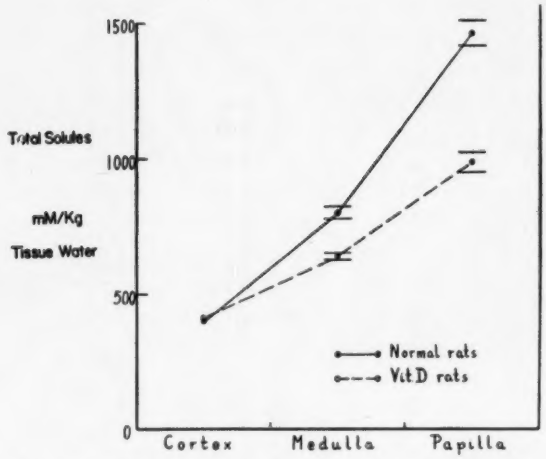



FIG. 1. THE CONCENTRATION OF "TOTAL SOLUTES," $2 \times (Na + K + NH_4) +$UREA, IN MEDULLA AND PAPILLA WAS CONSIDERABLY DECREASED IN RATS TREATED WITH VITAMIN D. The parallel lines indicate standard error of the mean.

that the calculated concentration of osmotically active solutes [1] in water of papillary and medullary tissue was significantly diminished in rats treated with vitamin D (Figure 1). The water content of medullary and papillary tissue was slightly increased in rats given vitamin D; this may have been a reflection of their polyuria.

The decrease in urinary osmolality induced by vitamin D was considerably greater, percentagewise, than was the fall in the concentration of sodium or of "total solutes" in the water of the renal papilla. The ratio of urinary osmolality to the papillary concentration of sodium and of "total solutes" was therefore significantly decreased in hypercalcemic animals (Figure 2).

## DISCUSSION

These experiments confirm findings of marked impairment of renal concentrating ability produced by hypercalcemia of several days' duration resulting from vitamin D intoxication. This change in renal function is associated with structural changes which are most prominent in the collecting ducts of the medulla but also involve the distal tubules and loops of Henle (2). The present data indicate that one reason for the decrease in maximal urinary concentration observed under these circumstances is a diminished ability of the kidneys to create and maintain a high concentration of sodium in the interstitial fluids of the medulla and papilla. Thus, the concentration of sodium in medullary and papillary tissue of hypercalcemic rats was significantly below that of normal controls, even though the concentration of sodium in the urine was greater than that of normals. The tendency of vitamin D-treated rats to lose sodium in the urine on a sodium-free diet further suggests that active reabsorption of sodium by some portion of the nephron was impaired by hypercalcemia and nephrocalcinosis.

It is of interest that the fall in maximal urinary osmolality produced by hypercalcemia was considerably greater than the decrease in sodium content or total solute concentration of the renal papilla or medulla. It is, of course, possible that changes in freezing-point of papillary tissue would not have paralleled the calculated change in concentration of tissue solutes. This might occur, for example, if the osmotic activity of cell solutes were altered owing to changes in protein-binding, or if the nature of the anions associated with sodium, potassium and ammonium were changed in the kidneys of animals treated with vitamin D. The calculated concentration of solutes in tissue water of the renal papilla in nor-

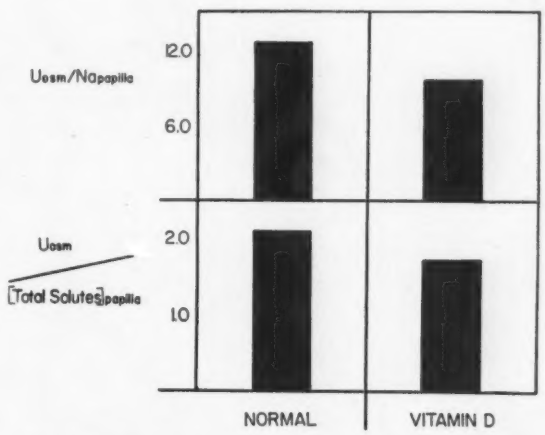



FIG. 2. THE RATIO OF MAXIMAL URINARY OSMOLALITY ($U_{osm}$) TO CONCENTRATION OF SODIUM AND "TOTAL SOLUTES" IN WATER OF THE RENAL PAPILLA WAS DIMINISHED IN NEPHROCALCINOTIC RATS.

[1] "Solute concentration" of renal tissue was calculated as $2 \times (Na + K + NH_4) +$ urea, where Na, K, $NH_4$ and urea are expressed as millimoles per kilogram of tissue water. It must be appreciated that this is only a rough approximation of the actual osmotic activity of tissue water.

mal hydropenic rats is considerably below urinary osmolality; this discrepancy is sharply diminished, however, as the terminal segment of the papilla is aproached (6). The final upturn in the concentration of sodium and total solutes at the papillary tip might be greatly reduced in nephrocalcinotic animals, without being detected in the present experiments. If that were the case, the fall in tissue sodium at the very tip of the renal papilla in rats treated with vitamin D might parallel their drop in maximal urinary osmolality, although analysis of the entire papilla would make it appear that the ratio of papillary sodium/urinary osmolality had increased.

An additional explanation for the changes illustrated in Figure 2 is that the back-diffusion of water from collecting ducts into the medullary and papillary interstitium may have been impaired in nephrocalcinotic rats, so that urinary osmolality fell more than did the concentration of sodium and other solutes in the water of the papilla. In this connection, it is of interest that the permeability to water of many cell membranes is diminished when calcium is added to their surrounding medium (7). The transfer of water out of the toad bladder in response to vasopressin is depressed when the concentration of calcium in fluid bathing the serosa is increased (8).

These results are similar to those obtained in potassium-deficient rats and dogs (6). In potassium depletion, ancillary evidence suggests that active reabsorption of sodium by the loops of Henle is unimpaired, and that the decreased concentration of sodium in the renal medulla and papilla results from interference with the reabsorption of sodium by the collecting ducts. Evidence bearing on this point in nephrocalcinotic animals might be obtained by studies of urinary dilution and by micropuncture of the distal tubule in rats intoxicated with vitamin D.

Acute decreases in glomerular filtration rate exceeding 30 per cent have been reported, by Levinsky, Davidson and Berliner (9), to reduce the sodium content of the renal medulla and papilla. Although it is not clear whether chronic depression of glomerular filtration results in similar changes, it is possible that alterations in glomerular filtration played some part in the reduction in sodium content of renal tissue noted in the present experiments.

Gill and Bartter (10) have reported that some human subjects with hypercalcemia, in whom a renal concentrating defect has been demonstrated, may reduce their urinary losses of sodium to the point of equilibrium between intake and output on a diet containing 9 mEq of sodium daily. This does not rule out the likelihood, suggested by the present studies, that sodium reabsorption in such patients is defective in some crucial segment of the nephron and that hyposthenuria is contributed to by a decreased concentration of sodium in the interstitial fluids of medulla and papilla.

## SUMMARY

1. The mechanism of hyposthenuria in hypercalcemia was investigated by analyzing renal tissue from rats intoxicated with vitamin D.

2. Vitamin D intoxication produced a significant fall in the content of sodium and urea of the renal papilla and medulla. The percentile decrease in the concentration of sodium and of total solutes in water of the renal papilla was not so great as the fall in maximum urinary osmolality. Renal conservation of sodium was impaired.

3. It is concluded that the impairment of renal concentrating ability observed in hypercalcemic rats results at least in part from impaired reabsorption of sodium by the renal tubules and a diminished ability to create and maintain a high concentration of sodium in the interstitial fluids of the medulla and papilla. The possibility that the permeability of the collecting ducts to the back-diffusion of water is reduced by vitamin D intoxication is discussed.

## ACKNOWLEDGMENT

The authors acknowledge gratefully the technical assistance of Mrs. Nadia Myketey and Mrs. Eva Taborsky.

## REFERENCES

1. Epstein, F. H. Calcium and the kidney. J. chron. Dis. 1960, **11**, 255.
2. Epstein, F. H., Rivera, M. J., and Carone, F. A. The effect of hypercalcemia induced by calciferol upon renal concentrating ability. J. clin. Invest. 1958, **37**, 1702.
3. Epstein, F. H., Beck, D., Carone, F. A., Levitin, H., and Manitius, A. Changes in renal concentrating ability produced by parathyroid extract. J. clin. Invest. 1959, **38**, 1214.

4. Carone, F. A., Epstein, F. H., Beck, D., and Levitin, H. The effects of transient hypercalcemia induced by parathyroid extract upon the kidney. Amer. J. Path. 1960, **36**, 77.
5. Smith, H. The fate of sodium and water in the renal tubules. Bull. N. Y. Acad. Med. 1959, **35**, 293.
6. Manitius, A., Levitin, H., Beck, D., and Epstein, F. H. On the mechanism of the impairment of renal concentrating ability in potassium deficiency. J. clin. Invest. 1960, **39**, 684.
7. Davson, H. Permeability to water *in* Permeability of Natural Membranes, H. Davson and J. F. Danielli, Eds. New York, Macmillan, 1943, chapter X.
8. Bentley, P. J. The effects of ionic changes on water transfer across the isolated urinary bladder of the toad *Bufo marinus*. J. Endocr. 1959, **18**, 327.
9. Levinsky, N. G., Davidson, D. G., and Berliner, R. W. Effects of reduced glomerular filtration on urine concentration in the presence of antidiuretic hormone. J. clin. Invest. 1959, **38**, 730.
10. Gill, J. R., Jr., and Bartter, F. C. On the impairment of renal concentrating ability in prolonged hypercalcemia and hypercalciuria in man. Clin. Res. 1959, **7**, 161.

# IMMUNOLOGICAL STUDIES WITH PLASMA EXPANDERS DERIVED FROM HUMAN PLASMA*

By PAUL H. MAURER† and D. SUBRAHMANYAM‡

*(From the Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa.)*

(Submitted for publication September 21, 1959; accepted December 24, 1959)

During the course of immunological testing of several proposed plasma expanders derived from human plasma, it was observed that the processing of the expanders created a new protein which was antigenic in humans. All the procedures for processing the human plasma incorporate heat treatment for 10 hours at 60° C in the presence of suitable stabilizers. This step is included to inactivate the virus of hepatitis (1–4).

The reprocessed, heat-treated plasma preparations fall into three classes: *1*) heated "whole" plasma from which little fibrinogen has been removed; *2*) partially fractionated materials from which gamma globulin, fibrinogen and some lipoproteins have been removed; *3*) albumin solutions. The stabilizers employed during the heating procedures were Na caprylate, Na acetyl tryptophanate, or dextrose.

In many cases these plasma expanders show evidence of major changes in their electrophoretic and ultracentrifugal patterns. In addition, some systemic reactions to infusions of the expanders have been noted (5). Because high molecular weight aggregates and possibly some denaturated albumin-globulin complexes are formed during the preparation of these expanders, studies were undertaken to learn whether or not they are antigenic in humans.

The present report deals with the techniques employed for studying the antigenicity of the expanders and with the methods for detecting the new protein antigen.

## MATERIALS AND METHODS

The materials that were studied for antigenicity in both rabbits and volunteers are summarized in Table I. The physicochemical data were furnished either by the manufacturers or by the Division of Biological Standards of the National Institutes of Health. Published procedures are available describing the preparation of SPPS[1] (6); the Mulford, Mealey and Welton material (7, 8); the Hoch and Chanutin preparation (9); the Cutter plasmanate (HTPF) (10); PPL (11); and PPF (12).

*1. Immunization of rabbits.* Rabbit antisera were obtained against the following preparations: Hyland heated plasma 230C-30; Mulford FFP9B; SPPS BD-21; Cutter heated plasma; USP heated plasma; and Chanutin albumin. The methods of immunization, dosages used and times of bleedings are summarized in Table II. All the preparations were tested after immunization of rabbits by the adjuvant technique. The adjuvant mixture was made by mixing an equal volume of plasma expander (diluted to about 5 per cent protein solution) with an equal volume of a mixture of Bayol F (9 parts) and Arlacel C (1 part) (13, 14). Dead mycobacteria (1 mg per ml) were also included. For immunization with alum-precipitated materials (SPPS and Mulford), the procedure of 4 injections per week for 4 weeks per course was followed, using a suspension of 1.5 mg protein per ml as described in Kabat and Mayer (15). All antisera were handled with sterile precautions and kept frozen until studied.

*2. Analysis of antisera.* As the main purpose of this phase of the study was to try to identify any "new antigens" produced during processing of human plasma, the Ouchterlony technique of agar diffusion as modified by Björklund was employed (16). Initially, the antisera were studied by placing the appropriate antiserum in the center well and varying dilutions of antigen in the outer wells. This arrangement was used to characterize the number and complexity of the antigen-antibody systems. The modification of Björklund employs,

* Presented in part at the XII Conference on Blood Cells and Plasma Proteins, November, 1957, Albany, N. Y. A preliminary report has appeared in Vox Sang. (Basel) 1956, 3, 72. Supported by a contract with the Office of the Surgeon General, Department of the Army Contract DA-49-007-MD-248. Publication 223, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh 13, Pa.

† Address after July 1, 1960: Seton Hall College of Medicine and Dentistry, Jersey City, N. J.

‡ Present address: University of Western Ontario, Faculty of Medicine, London, Ontario, Canada.

[1] The abbreviations used are: SPPS—stabilized purified protein solution; HTPF—heat-treated protein fraction; PPL—pasteuriserte plasma protein-Lösung; PPF—plasma protein fraction.

instead of agar, agar mixed with normal human serum to a final concentration of 25 per cent by volume. When antiserum against normal human serum proteins is placed in the center well, free diffusion of the many antibodies is prevented by the tremendous excess of antigen around the center well. Only antibodies that are not formed against normal human serum proteins can diffuse freely. In the presence of the appropriate concentration of "new antigen," a band or bands will appear, depending upon the number of precipitating systems present.

Each antiserum was studied by this technique. The only system found to be positive was that of the Chanutin albumin-antiChanutin albumin serum.

Additional agar diffusion studies (in the presence of normal human serum) were made with Chanutin albumin antisera in the center well and dilutions of all the other plasma preparations listed in Table I in the outer wells. Other materials studied with this antiserum were Hyland HSA (4 preparations) and the following subfractions of serum: Fr. II, III, IV-1, IV-4, IV-5, IV-8, IV-9, V, VI, ceruloplasmin and $\beta_1$ metal combining globulin. Other studies employed serum-agar plates which were made with Hyland and USP heated and unheated plasmas.

The antiChanutin serum was calibrated by the quantitative immunochemical procedures developed by Heidelberger and co-workers (15). The calibrated antiserum was used to compare several serum albumins with the Chanutin preparation and also to determine the appropriate concentration of albumin to use for absorbing the antialbumin antibodies. The antiserum that was absorbed at equivalence with HSA was studied again in agar diffusion in the presence of normal human serum and the various human plasma preparations and also by the quantitative precipitin technique with Chanutin albumin. Two other antisera produced against placental albumin and Hyland Fr. V were included in the study. These were investigated in the manner described above for the antiChanutin serum.

*3. Electrophoretic studies. a) Continuous filter paper electrophoresis*[2] (17, 18). The following samples were fractionated on a Spinco hanging paper electrophoretic apparatus kept in a cold room: Chanutin albumin, Hy-

[2] The authors wish to thank Dr. William Merchant and Mr. Thomas Giglotti of the Department of Research Medicine, VA Hospital, Pittsburgh, Pa., for carrying out these separations.

TABLE I

*Some properties of heated and unheated human plasma preparations*

| Type of material | Preparation | Protein conc. | Stabilizer added | Electrophoretic data | | | | | | Sedimentation data* (component) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Alb. | Fib. | $\alpha_1$ | $\alpha_2$ | $\beta_1$ | $\gamma$ | I | II | III |
| | | % | | % | | | | | | | | |
| Whole plasma | Hyland Labs 230C30 Unheated | | | 65 | ←12→ | | | 11 | 12 | | | |
| | Heated†‡ | 5.2 | 5% dextrose | 44 | ←47→ | | | 3 | 6 | 17.4(2.4), | 6.7(16.6), | 4.0(81) |
| | USP P52-0003 Unheated | | | 55 | ←28→ | | | | 17 | | | |
| | Heated†‡ | 4.7 | 5% fructose | 35 | ←55→ | | | | 10 | 27.5(4) | 5.7(4) | 4.3(86) |
| Partially fractionated plasma | SPPS BD-21 Unheated | | | 65.2 | | 6.2 | 10.0 | 14.1 | 4.5 | | | |
| | Heated†‡ | 4.5 | 0.008 M -caprylate | 65.6 | | 4.2 | 25.2 | 4.6 | 1.8 | | | |
| | Mulford FFP9B†‡ | 3.6 | 0.008 M Na acetyl tryptophanate | 81 | | | ←19→ | | | | 4.0(100) | |
| | Cutter plasmanate (HTPF) (1242) | 4.9 | 0.004 M acetyl tryptophanate 0.004 Na caprylate | 88.5 | | ←7.8→ | | 3.7 | | | 4.0(100) | |
| | Swiss Red Cross (PPL)§ T45 | | 0.004 M Na caprylate +5% glucose | 66–70 | | 30–34 | | | | | 4.0(98) | |
| | Auerswald Austrian-prepn. PPF (211356) | 3.9 | | 88 | ←7→ | | | 5 | 0 | | | |
| Albumins | Chanutin 1309† | 24 | 0.02 M acetyl tryptophanate + 0.02 M Na caprylate | 100 | | | | | | | 3 components | |
| | 1334 | 25 | | 100 | | | | | | | | |
| | Hyland HSA 490B4 | 25 | Same as above | 100 | | | | | | | | |

* Values in parentheses refer to per cent of respective component present in mixture.
† Preparation studied for antigenicity in volunteers.
‡ Preparation studied for antigenicity in rabbits.
§ Prepared by desalting of plasma (ion exchange treatment).

TABLE II

*Procedures used in immunization of rabbits*

| Prepn. | Method of immunization | Rabbits | Dosage employed | Dates of bleedings |
|---|---|---|---|---|
| | | *no.* | *mg.* | |
| Hyland 230C-30 | Complete adjuvants* | 3 | 26, 26† | Weekly, 6 wks after 2nd inj. |
| | | | 26‡ | Weekly, 5 wks after 3rd inj. |
| USP | Complete adjuvants | 4 | 18,† | Weekly, 5 wks after 2nd inj. |
| | | | 18,‡ | Weekly, 5 wks after 3rd inj. |
| SPPS | Alum ppt—2 courses | 4 | 50 mg/course | Wk after last inj. of each course |
| | Complete adjuvant | 4 | 20, 20† | Weekly for 6 wks after 2nd inj. |
| Mulford | Alum ppt—2 courses | 4 | 50 mg/course | Wk after last inj. of each course |
| | Complete adjuvants | 4 | 20, 20† | Weekly, 4 wks after 2nd inj. |
| | | | 20‡ | Weekly, 4 wks after 3rd inj. |
| Cutter plasmanate (HTPF) | Complete adjuvants | 5 | 25, 25† | Weekly, 3 wks |
| | | | 25‡ | Weekly, 4 wks |
| Chanutin | Complete adjuvants | 4 | 25 | Weekly, 5 wks |

* Complete adjuvants refer to mixture continaing dead mycobacteria.
† Interval of 2 weeks between 1st and 2nd injection.
‡ Third injection given after last bleeding of previous course.

land heated and unheated plasmas. The samples were dialyzed overnight in the cold against veronal buffer (pH 8.6; $\mu = 0.02$) before application to the curtain. The entire apparatus was stabilized overnight by application of buffer to the curtain (S & S paper no. 470). Fractionation was performed at 50 ma and 720 v. The feeding rate was adjusted to handle 15 ml of protein solution per 18 hours. The circulating buffer was further refrigerated by cycling through a cold bath. The temperature of the curtain never exceeded 18° C. The material which was collected in 32 tubes was diluted with about 5 to 10 ml buffer; 0.5 ml aliquots were heated for 20 minutes in a boiling water bath with 0.5 ml of ninhydrin. Following cooling, 4 ml of 50 per cent propanol was added, and the color that developed was read at 540 mμ and plotted. The various fractions were then lyophilized and reconstituted for agar diffusion studies.

b) *Immunoelectrophoresis.* Immunoelectrophoretic studies as described by Grabar and Williams (19, 20) were performed with antiChanutin albumin serum unabsorbed and absorbed with crystalline HSA and with normal human serum, respectively. An Arthur H. Thomas paper electrophoretic assembly was adapted to immunoelectrophoresis by employing glass plates and a 3 to 4 mm layer of Noble agar made with veronal buffer (pH 8.3; $\mu = 0.05$). The plates contained *1*) two circular wells in which Chanutin albumin was placed and then subjected to electrophoresis; *2*) three rectangular wells to which the appropriate antiserum was added for development of the antigen-antibody bands. Diffusion of antiserum and antigen was allowed to proceed for several days in a cold room in a sealed system to prevent excessive loss of water and drying of the agar plates.

*4. Immunization of volunteers.* Five of the preparations were studied in man as follows: 50 ml of blood was obtained from healthy medical students before skin testing with the appropriate material, including saline as a control. Skin reactions were read 15 minutes later. Twelve volunteers divided into 3 groups of 4 were tested with each material. Each group of 4 volunteers then received an intramuscular injection of 5, 10 or 20 mg of protein per injection 5 times over a 7 to 10 day period. Bleeding and skin testing of students were repeated approximately 10 days and 3 to 4 weeks after the last injection. Reactions of the students were reported and recorded appropriately.[3] Some of the volunteers were retested 2 months after the last injection; 500 ml bleedings from several of the subjects who showed some reactions were also obtained for further study. All serum samples were handled with sterile precautions and, in addition, "Merthiolate" (0.01 per cent) and phenol (0.25 per cent) were added.

*5. Analysis of human sera.* All the sera obtained were analyzed for precipitins, both by the quantitative micro precipitin technique of Heidelberger and MacPherson (21, 22) and by various agar diffusion procedures.

a) *Micro precipitin technique.* Before analysis, the sera were centrifuged overnight in the cold as described by Maurer (23). Three ml portions of pre- and post-immunization sera were measured into 8 ml conical centrifuge tubes calibrated at the 2.5 ml mark. Concentrations of the appropriate preparation containing from 3 μg N to 700 μg N were added to the sera. The tubes were mixed, capped, placed in a water bath at 37° C for 1 hour and then in the refrigerator for 10 to 14 days, the contents of the tubes being mixed daily. The tubes were then centrifuged and the precipitates washed and analyzed as described previously. Additional antigen

[3] A more detailed study is available in the files of the National Research Council, Committee on Plasma. A complete summary of the reactions will be furnished upon request.

was added to the supernatants and treated as above. Appropriate controls of serum plus saline and of antigen alone were included.

b) *Agar diffusion studies.* The sera were analyzed by the Oudin (24), Preer (25), and Ouchterlony (26, 27) techniques of agar diffusion as follows. The concentrations of overlayering "antigen" employed in the Oudin procedure were 200 μg N, 500 μg N and 1 mg N per ml. With the Preer technique, undiluted serum (0.02 ml) was overlaid with agar (0.6 per cent), which was in turn overlaid with undiluted "antigen" or with a 1:2, 1:8, 1:32, 1:128, or 1:512 dilution of the "antigen." When the Ouchterlony technique was employed, it was necessary to prepare the agar plates with glycine and veronal buffer as described by Halbert, Swick and Sonn (28). This procedure was employed to eliminate the haze that forms around undiluted human serum in the presence of buffered agar alone. Undiluted sera were studied in the presence of undiluted "antigen" or of a 1:2, 1:8, 1:32, 1:128, or 1:512 dilution of "antigen." Observations were made daily for at least 2 weeks.

## RESULTS

*1. Rabbit antisera.* The only antiserum which, in agar diffusion studies, indicated the presence in the plasma expanders of a new component not present in normal human serum was the Chanutin albumin rabbit antiserum. Dilutions of Chanutin albumin reacting with antiChanutin albumin serum produced a definite antigen-antibody band in the presence of serum-agar mixtures (Figure 1). Increasing the concentration of human serum in agar up to 50 per cent did not remove the band. This same band persisted even when the anti-albumin had been removed from the antiserum by

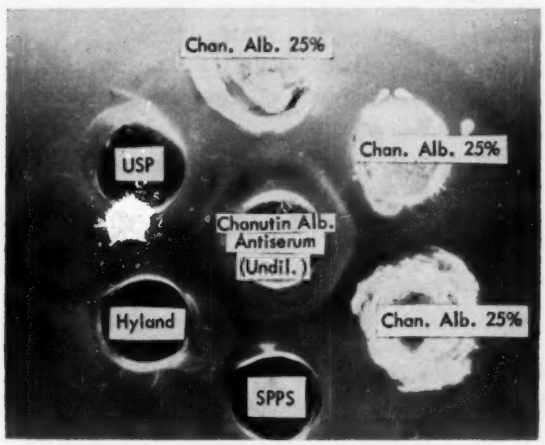



FIG. 1. AGAR DIFFUSION PATTERNS OF REACTIONS BETWEEN PLASMA EXPANDERS AND RABBIT ANTICHANUTIN ALBUMIN SERUM.

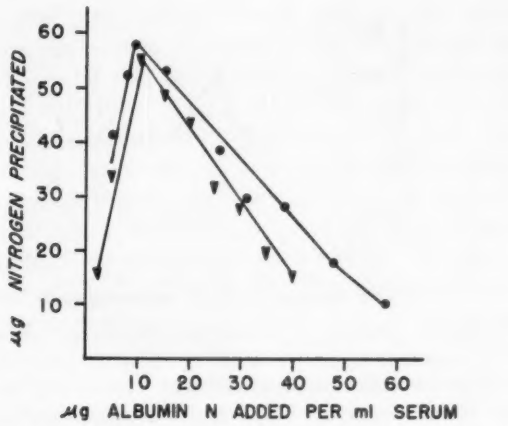



FIG. 2. PRECIPITIN CURVES OF CHANUTIN ALBUMIN ANTISERA (1:8 DILUTION) WITH CHANUTIN ALBUMIN (●) AND CRYSTALLINE HSA (▼).

absorption. Although none of the other plasma preparations showed such a reaction with its respective antiserum, many of them reacted with the antiChanutin serum. The SPPS, USP, Mulford and Hyland heated plasma preparations all gave joining bands with the Chanutin preparation (Figure 1) but did not react with their homologous antisera even when the samples had been concentrated threefold. The unheated USP and Hyland 230C-30 did not give bands. The heated and unheated Cutter preparations, Auerswald and normal Fr. V albumins from various sources did not give a positive reaction with the Chanutin antiserum. Serum agar plates made with heated Hyland or USP plasma inhibited the formation of the band, whereas the plates made with the unheated preparations did not.

The quantitative immunochemical study of the reaction of the Chanutin albumin with calibrated anti-HSA serum and the reaction of various albumins with antiChanutin sera (Figure 2) revealed no significant immunochemical differences. The antiserum against the Chanutin albumin preparation contained antibodies directed against many other proteins. When studied by agar diffusion (in the absence of normal human serum), positive reactions were obtained between the Chanutin antiserum and the following subfractions of serum: Fr. II, III, IV-1, IV-4, IV-5, IV-8, IV-9, VI, $\beta_1$ metal binding globulin and ceruloplasmin.

The data presented in Figure 3 indicate that the Chanutin albumin antiserum does contain anti-

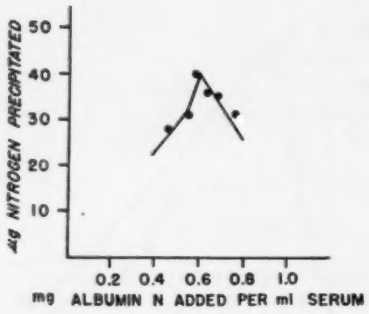



FIG. 3. PRECIPITIN CURVE OF CHANUTIN ALBUMIN ANTISERA ABSORBED AT EQUIVALENCE WITH CRYSTALLINE HSA WITH CHANUTIN ALBUMIN.

bodies precipitable by Chanutin albumin even after absorption at equivalence with crystalline HSA. This finding is further evidence of the heterogeneity of the Chanutin albumin preparation. If we assume an approximate ratio at equivalence of (antibody N)/(antigen N) of 4.5, it can be estimated that the Chanutin albumin contains at least 1 per cent of other proteins.

When the various fractions obtained by continuous paper electrophoresis from the Chanutin, Hyland and USP heated and unheated plasmas were allowed to react with antiChanutin serum in the presence of normal human serum and agar, the Chanutin and heated plasmas of USP and Hyland contained the new antigen. The antigen was located only in the fastest moving fractions of the albumin. Bands in agar diffusion were obtained with Hyland fractions 27, 28, USP fractions 29, 30 and Chanutin albumin fractions 28 to 30. Figure 4 shows the type of separations achieved and where the new antigen was located. The results obtained above were also

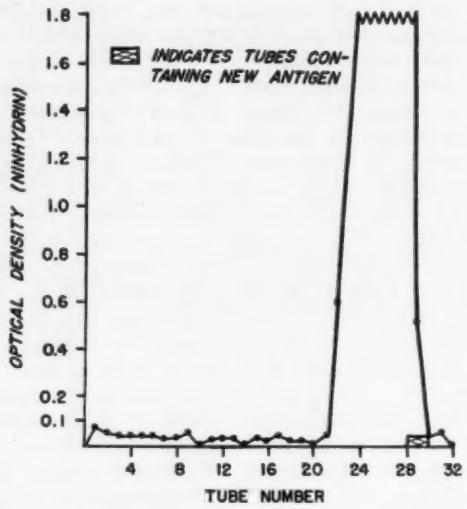



FIG. 4. CONTINUOUS PAPER ELECTROPHORETIC SEPARATION OF CHANUTIN ALBUMIN, 5 PER CENT (BARBITAL BUFFER pH 8.6, $\mu = 0.02$, 50 MA, 720 V).

confirmed by immunoelectrophoretic analysis. The antigen involved in the reaction with the absorbed antiChanutin serum was shown to be at the leading edge of the albumin area.

*2. Skin testing of volunteers.* With all the preparations studied, some cutaneous reactions were observed at the time of testing. There was only one marked systemic reaction following the skin testing. Skin reactions were considered positive when the wheal and erythema measured 10 mm or greater. In one individual, a marked delayed reaction was evident 24 hours after skin testing. A summary of the number of reactions with each material is presented in Table III.

*3. Immunochemical analysis of human sera.* Of all the sera analyzed by quantitative immuno-

TABLE III

*Summary of reactions in volunteers injected with plasma preaparations*

| Material injected | No. of volunteers | No. of positive skin reactions | | | Systemic reactions |
|---|---|---|---|---|---|
| | | Pre-imm. | 10 Days after last inj. | 3 Wks after last inj. | |
| SPPS BD-21 | 11 | 0 | 4 | 2 | 1 (Hives, dry and irritated throat) |
| Hyland 230-C-30 | 11 | 1 | 1 | 2 | 1 (Leg pains during immunization) |
| Mulford FFP9B | 12 | 2 | 0 | 2 | 0 |
| Cutter plasmanate | 12 | 2 | 5 | 5 | 0 |
| USP heated plasma | 12 | 0 | 5 | 5 | 0 |

chemical techniques, only serum from one individual in the SPPS group gave a definite precipitate with the antigen. After removal of complement with a specific precipitate, a precipitate of 2.1 μg N per ml was obtained. This value, which is at the limit of significance of the colorimetric method (29), includes not only "antibody" but also "antigen." What fraction of the total SPPS added is precipitated as "antigen" is not known. A subsequent bleeding obtained three weeks later showed a decrease in total N precipitated.

None of the agar diffusion techniques employed indicated the presence of antibody when the human sera were tested. This result was to be expected, as the level of antibody detectable by these techniques is usually of the order of several micrograms of antibody N (30).

## DISCUSSION

The effect of heat treatment on the electrophoretic and immunological properties of serum and plasma has been known for some time (31–34). The extent of alteration in the physicochemical properties appears to be related to the complexing that occurs between molecules of albumin and of gamma globulin. In addition, increased heating leads to denaturation and to changes in the specificity of the serum proteins (35). The addition of appropriate stabilizers can reduce considerably the extent to which these reactions occur.

In the studies discussed here, an antigen not present in unheated plasma has been demonstrated in heated plasma. Although antibodies against the new antigen were produced in rabbits only following immunization with the Chanutin albumin, this antiserum could detect the antigen in many other heat-treated preparations of plasma.

The nature of this new antigen is of some interest. Originally it was thought that the new antigen would be associated with albumin-globulin complexes which were produced during the heating process. However, electrophoretic fractionation and immunoelectrophoresis indicated that the antigen was associated with the fast-moving fractions of albumin. The new antigen was definitely produced by heating (10 hours, 60° C) since it was not present in the unheated preparations. The joining of antigen-antibody bands indicated the similarity of this antigen in all the preparations. Some preparations which were exposed to the same heating procedure did not exhibit this new antigen, i.e., Cutter plasmanate (HTPF), Auerswald PPF, and several albumin preparations. This fact indicated that heating alone did not produce the new antigen. Although this "antigen" moves as a fast albumin, it is sufficiently different from albumin so that antibodies produced against it are not removed by normal human serum albumin (no cross reaction). The new antigen may be an albumin which has been partially hydrolyzed during heating. It was not possible to separate the new component in a Spinco ultracentrifuge (Preparative Model L) when run at 35,000 rpm for three hours. This observation indicates that the new antigen is not exceptionally heavy. Also, in the agar diffusion studies, the bands appeared close to the antibody well, possibly indicating that the material had a high diffusion constant.

One can only speculate about the interpretation of the data obtained with the immunization of volunteers. With one exception, no precipitating antibody was detectable in the human sera from the volunteers injected with the heated preparations. Because the nature of the response evoked by the injection of the solution of protein was frequently of a character making it difficult to distinguish between an immediate irritative response and one of hypersensitivity, a complete interpretation of the cutaneous reactions is not possible. However, cutaneous reactions such as itching at the skin test site during the course of immunization or at the ten day or three week testing dates, urticaria, a hot and irritated throat, and the "delayed reaction" of one subject suggest that some immunological phenomena may be responsible for the symptoms observed. The nature of the "antigen" responsible for the cutaneous and systemic reactions is completely unknown. It is not known whether the new antigen detected in many of these preparations with the rabbit antiserum (Chanutin albumin) is also the "antigen" responsible for the reactions in humans. It may well be that the artificially produced albumin-globulin complexes present in some preparations, or traces of denatured proteins in others, may be the antigen. That some individuals may react to normal human serum proteins that are different from their own is also plausible. Simi-

lar situations do exist in rabbits (36–38). Until we know more about the interpretation of the cutaneous reactions of individuals to highly purified substances, it is difficult to assess completely or adequately the reaction to materials consisting of a mixture of antigens. One can only speculate, therefore, about reasons for the cutaneous reaction to the materials employed. *1*) Perhaps we are dealing with a classical "reagenic" type of antibody which gives positive skin reactions. The "antibody" may be present in such low concentrations that precipitation cannot be obtained. *2*) The other possibility is that, in some instances, we may be dealing with "delayed hypersensitivity" to altered serum proteins.

## SUMMARY

It has been demonstrated that many heated preparations of plasma contain a new antigen not present in the unheated materials. The usefulness of agar diffusion techniques in detecting this new component was shown. Immunization of volunteers with the human plasma preparations in several instances led to the appearance of reactions. The difficulty in attributing the reactions to any one antigen, as well as in interpreting the significance of the reactions, has been discussed.

## ACKNOWLEDGMENTS

The authors wish to acknowledge the assistance of the following individuals: Miss Christine Vance for technical assistance; Dr. H. C. Mansmann, for supervising the skin testing of the volunteers; Mr. William Arnold and Mr. Al Nordin, for help in bleeding of medical volunteers.

The cooperation and assistance of Drs. Margaret Sloan and James Weisiger of the National Research Council and the various manufacturers and individuals who supplied materials are gratefully acknowledged.

## REFERENCES

1. Murray, R., Diefenbach, W. C. L., Ratner, F., Leone, N. C., and Oliphant, J. W. Carriers of hepatitis virus in the blood and viral hepatitis in whole blood recipients. 2—Confirmation of carrier state by transmission experiments in volunteers. J. Amer. med. Ass. 1954, **154**, 1072.
2. Murray, R., and Diefenbach, W. C. L. Effect of heat on the agent of homologous serum hepatitis. Proc. Soc. exp. Biol. (N. Y.) 1953, 84, 230.
3. Murray, R. Conference on Implication of New Knowledge about Protein, Protein Enzymes and Cells. Protein Foundation, Cambridge, Mass., January 14, 1952, p. 82.
4. Gellis, S. S., Neefe, J. R., Stokes, J., Jr., Strong, L. E., Janeway, C. A., and Scatchard, G. Chemical, clinical and immunological studies on the products of human plasma fractionation XXXVI. Inactivation of the virus of homologous serum hepatitis in solutions of normal serum albumin by means of heat. J. clin. Invest. 1948, **27**, 239.
5. Reports presented to National Research Council, Division of Medical Sciences 1955–1958.
6. Pennell, R. B. Conference on New and Modified Procedures for the Preparation of Stable Proteins from Human Plasma. Harvard Medical School, April 24, 1953.
7. Mulford, D. J., Mealey, E. H., and Welton, L. D. Preparation of a stable human plasma protein solution. J. clin. Invest. 1955, **34**, 983.
8. Mulford, D. J., and Mealey, E. H. Heat stability of protein solutions obtained from human plasma by different ethanol concentrations and temperatures. J. clin. Invest. 1956, **35**, 1213.
9. Hoch, H., and Chanutin, A. Albumin from heated human plasma 1. Preparation and electrophoretic properties. Arch. Biochem. 1954, **51**, 271.
10. Hink, J. H., Jr., Hidalgo, J., Seeberg, V. J., and Johnson, F. F. Preparation and properties of a heat treated human plasma protein fraction. Vox Sang. (Basel) 1957, **2**, 174.
11. Nitschmannn, H., Kistler, P., Renfer, H. R., Hässig, A., and Joss, A. A heat stable human plasma protein solution obtained by desalting (PPL). Vox Sang. (Basel) 1956, **1**, 183.
12. Auerswald, W., Doleschel, W., and Szegvari, G. Physico-chemical and immunological comparison of stabilized, dissolved plasma protein fractions (PPF) before and after inactivation by heat. Anesthetist. In press.
13. Ward, R., Rader, D., Lipton, M. M., and Freund, J. Formation of neutralizing antibody in monkeys injected with poliomyelitis virus and adjuvants. Proc. Soc. exp. Biol. (N. Y.) 1950, **74**, 536.
14. Salk, J. E., and Youngner, J. S. Antibody in yolk of eggs and in serum from hens inoculated with viruses of influenza and poliomyelitis. Fed. Proc. 1950, **9**, 390.
15. Kabat, E. A., and Mayer, M. M. Experimental Immunochemistry. Springfield, Ill., Charles C Thomas, 1948.
16. Björklund, B. Immunologic studies of rabbit bone marrow with the aid of gel diffusion and serologic techniques. Int. Arch. Allergy 1953, **4**, 340.
17. Durrum, E. L. Continuous electrophoresis and ionophoresis on filter paper. J. Amer. chem. Soc. 1951, **73**, 4875.
18. Lewis, J. H., Walters, D., Didisheim, P., and Merchant, W. R. Application of continuous flow electrophoresis to the study of the blood coagulation proteins and the fibrinolytic enzyme system.

I. Normal human materials. J. clin. Invest. 1958, **37**, 1323.

19. Grabar, P., and Williams, C. A. Méthode permettant l'étude conjugée des propriétes électrophoretiques et immunochimiques d'un mélange de protéines; application au sérum sanguin. Biochim. biophys. Acta 1953, **10**, 193.
20. Grabar, P., and Williams, C. A., Jr. Méthode immuno-électrophorétique d'analyse de mélanges de substances antigéniques. Biochim. biophys. Acta 1955, **17**, 67.
21. Heidelberger, M., and MacPherson, C. F. C. Quantitative micro-estimation of antibodies in the sera of man and other animals. Science 1943, **97**, 405.
22. Ibid. 1943, **98**, 63.
23. Maurer, P. H. Antigenicity of oxypolygelatin and gelatin in man. J. exp. Med. 1954, **100**, 497.
24. Oudin, J. Methods in Medical Research. Chicago, Yearbook Publishers, Inc., 1952, vol. 5, p. 335.
25. Preer, J. R., Jr. A quantitative study of a technique of double diffusion in agar. J. Immunol. 1956, **77**, 52.
26. Ouchterlony, Ö. Antigen-antibody reaction in gels. Ark. Kemi Min. Geol. 1949, **B26**, 1.
27. Ouchterlony, Ö. Antigen-antibody reaction in gels II. Factors determining the site of the precipitate. Ark. Kemi Min. Geol. 1949, **1**, 43.
28. Halbert, S. P., Swick, L., and Sonn, C. The use of precipitin analysis in agar for the study of human streptococcal infections II. Ouchterlony and Oakley technics. J. exp. Med. 1955, **101**, 557.
29. Kabat, E. A., and Berg, D. Dextran—An antigen in man. J. Immunol. 1953, **70**, 514.
30. Finger, I., and Kabat, E. A. A comparison of human antisera to purified diphtheria toxoid with antisera to other purified antigens by quantitative precipitin and gel diffusion techniques. J. exp. Med. 1958, **108**, 453.
31. Van der Scheer, J., Wyckoff, R. W., and Clark, F. H. An electrophoretic study of heated horse-serum. J. Immunol. 1941, **40**, 39.
32. Kleczkowski, A. The formation of protein complexes in heated solutions of rabbit serum proteins. Brit. J. exp. Path. 1941, **22**, 188.
33. Kleczkowski, A. Effect of heat on flocculating antibodies of rabbit antisera. Brit. J. exp. Path. 1941, **22**, 192.
34. Bawden, F. C., and Kleczkowski, A. The effects of heat on the serological reactions of antisera. Brit. J. exp. Path. 1942, **23**, 178.
35. Furth, J. Antigenic character of heated protein. J. Immunol. 1925, **10**, 777.
36. Oudin, J. "L'allotypie" de certains antigènes protéidiques du sérum. C. R. Acad. Sci. (Paris) 1956, **242**, 2606.
37. Oudin, J. Réaction de précipitation spécifique entre des sérums d'animaux de même espèce. C. R. Acad. Sci. (Paris) 1956, **242**, 2489.
38. Dray, S., and Young, G. O. Differences in the antigenic components of sera of individual rabbits as shown by induced isoprecipitins. J. Immunol. 1958, **81**, 142.

# REVUE FRANÇAISE D'ÉTUDES CLINIQUES ET BIOLOGIQUES

MARCH, 1960

Vol. V, No. 3

Editorial

L'Alcaptogenèse, Problème non Résolu. P. GONNARD.

Original Articles

Étude du Métabolisme du Calcium Chez le Rat à l'Aide de Calcium 45. I. Le Rachitisme Expérimental. G. MILHAUD, W. REMAGEN, A. GOMES DE MATOS ET J.-P. AUBERT.

Étude de la Formation du Tissu Conjonctif Chez le Rat. CH. M. LAPIÈRE et H. VAN CAUWENBERGE.

Action Curative de la Moelle Osseuse Isologue Chez les Rats Ayant Recu une Dose Létale de Myleran. Le Rapport Entre le Nombre de Cellules de la Moelle Osseuse Transfusée et la Survie. A. DUNJIC et J. MAISIN.

Anatomapathologie Expérimentale. I. Néphropathies Chroniques Ascendantes Expérimentales Chez le Lapin. B. ANTOINE et H. DE MONTÉRA.

Original Notes

Influence de l'Histamine sur l'Hypertrophie Compensatrice du Foie Suivant une Hépatectomie des Deux Tiers Chez le Rat Blanc. R. MOLIMARD.

Détermination Quantitative et Qualitative des Sulfates de Stéroïdes à l'Aide du Bleu de Méthylene. I. Microdosage des Stéroïdes Sulfates. O. CRÉPY.

Détermination Quantitative et Qualitative des Sulfates de Stéroïdes à l'Aide du Bleu de Méthylene. II. Détection des Sulfates de Stéroïdes en Chromatographie sur Papier. O. CRÉPY et O. JUDAS.

Protéines et Enzymes Tissulaires. Une Étude par Électrophorèse en Gel d'Amidon. J. DE GROUCHY.

Influence d'Agents Complexants sur le Développement de la Fibrose Expérimentale par Silice Chez le Rat. H. DANIEL-MOUSSARD et A. COLLET.

Étude de l'Effet Bridge par les Tests Combinés de Thromboplastinoformation et de Thrombinoformation. Action des Thromboplastines Partielles (Incompletes) sur des Substrats Manquant d'un Facteur Thromboplastique. G. MAYER, S. MAYER et R. WAITZ.

Revue Française d'Etudes Cliniques et Biologiques n° 3 (Mars), Vol. V.

General Review

La Biosynthese des Protéines. Nouveaux Aspects du Problème. II. Synthese de Protéines Spécifiques. J. KRUH.

Technical Note

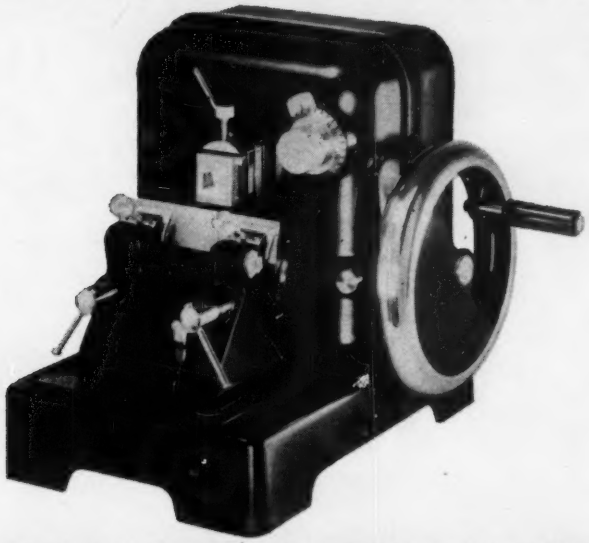
Dosage des Catécholamines Urinaires. M. MASSON.

Subscription rate, $19.00

Special rate for interns and residents, $13.00

EDITIONS MEDICALES FLAMMARION, 22, rue de Vaugirard, PARIS 6ème. France.

*for dependable, precision microtome sectioning*



# LEITZ LARGE MINOT ROTARY MICROTOME

The Large Minot Rotary Microtome (#1212) embodies traditional Leitz quality in a heavy design that insures the utmost rigidity and freedom from vibration. This microtome is ideally suited for rapid, accurate, serial sectioning of biological and pathological specimens and for industrial applications such as examination of textile fibers.

- precision micrometer mechanism permits selection of cutting thickness between 1 and 25 microns
- rigid knife blocks with rotating knife clamps, heavy ball-and-socket device for object clamp and paraffin stage
- ball-and-socket clamp permits rapid positioning of the specimen in any direction
- inclination of the knife readily adjustable as required
- adjustable knife block available for producing paraffin sections with obliquely positioned knife
- conveyor belt available for receiving series of sections

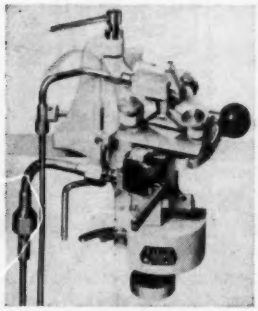
*Write today for illustrated brochure #53-8, "Leitz Microtomes."*

Leitz

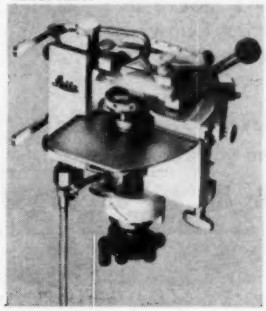
E. LEITZ, INC., 468 PARK AVENUE SOUTH, NEW YORK 16, N. Y.
Distributors of the world-famous products of Ernst Leitz G. m. b. H., Wetzlar, Germany—Ernst Leitz Canada Ltd.
LEICA CAMERAS · LENSES · PROJECTORS · MICROSCOPES · BINOCULARS

Other Leitz Microtomes:

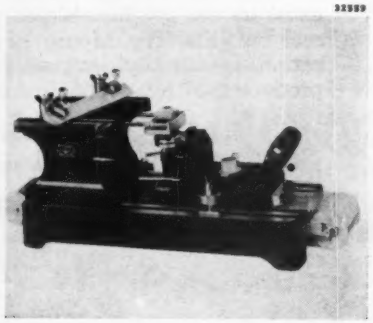
Freezing Microtome (#1213) for sections from 5 to 10 microns in 5-micron intervals.



Large Freezing Microtome (#1310) for sections from 2.5 to 50 microns in 2.5-micron intervals.



Base Sledge Microtome (#1300) for large and/or very hard sections from 1 to 20 microns thick.



*Properly nourished breeding stock, and an adequate milk supply, can help you*

# produce large litters per female!

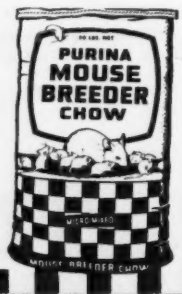
Purina Mouse Breeder Chow helps you produce mice in many ways.

Improved reproduction, through the use of a ration specifically designed for breeding mice, is to be expected. Then, less obvious benefits of palatability . . . which encourages baby mice to eat at an early age and thereby achieve spectacular growth . . . and increased milk production, which helps combat infantile death . . . can be added.

Results of these combined advantages have increased yearly production of mice per female as much as 100% in controlled tests.

From a cost standpoint, even the addition of just *one* extra mouse per female per year makes this product a real bargain. Try Mouse Breeder Chow. Let this new ration, specifically designed for breeding mice, help you produce healthy, prolific colonies.

- designed specifically for breeding mice
- packed in new, polyethylene-lined bag for maximum protection from contamination
- produced in popular 3/16 to 5/16 inch oval pellet to provide maximum bite surface and minimum waste




**RALSTON PURINA COMPANY**

**with 5,000 Dealers to serve you coast to coast**

# over 750 COULTER COUNTER® installations!

**Non-optic automatic cell counting and cell size analyzing provides unprecedented accuracy, speed for counting and sizing red cells, white cells, tissue cultures, bacteria**

**SEE US IN BOOTH #1**
**Steel Pier—Atlantic City**
**May 1–3**

***The Coulter Counter's operational abilities include:***

- Sample capacity exceeds 60 counts per hour on a production basis.
- Thoroughly field tested in leading institutions for routine laboratory tests and research.
- As little as .02 ml. blood samples for both red and white count.
- Sensitivity extends to particles smaller than 1 micron.
- Counts in excess of 6,000 individual cells per second!
- Each count equivalent in number of cells counted to average of 100 chamber counts . . . reduces sampling error by factor of approximately 10.
- Unit takes its own precisely metered sample from sample beaker to eliminate counting chamber errors.
- Oscilloscope display provides immediate information on relative cell size and relative cell size distribution.
- Threshold level control provides means of rapidly obtaining complete cell size distribution data.
- Oscilloscope display provides check of circuit performance . . . mechanical design is simple—result: highest reliability!

**Coulter Electronics (7)**
**5227 N. Kenmore Avenue/Chicago 40, Illinois**

☐ Please send complete details describing the Coulter Counter.
☐ Please get in touch with me in reference to a demonstration in my office or laboratory.

Name.....

Association.....
(hospital, school, etc.)

Address.....

City.....Zone.....State.....Phone.....

***Send in this coupon today!***

## COULTER ELECTRONICS

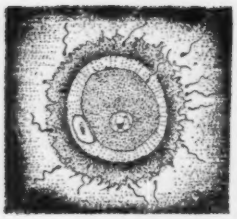
TRADEMARK

**5227 N. Kenmore Avenue**
**Chicago 40, Illinois**

**Call Collect: UPtown 8-2942**

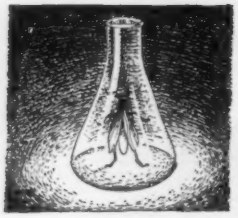
# *IT HAPPENED THIS MONTH...*

*a glance at yesterday in relation to today*



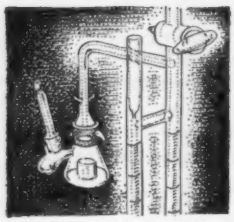
IN APRIL—(1900)—there is a review in Nature by "J. A. T." on the current controversy on the mechanism of fertilization.[1] Yves Delage is reported as suggesting that the pronucleus of the ovum is unnecessary. "The female pronucleus is perhaps useful in securing for the embryo the advantages of amphimixis, but it is not useful in fertilization nor necessary for the development of the parts of the organism." This contrasts with Jacques Loeb, who states, "The unfertilized egg of the sea urchin contains all the elements for the production of a perfect pluteus. . . . The spermatozoon may, however, carry in addition a number of enzymes or other material. But the ions and not the nucleins in the spermatozoon are essential to the process of fertilization."

*Six decades later, geneticists and embryologists do not question the importance to fertilization of both egg and sperm "nucleins." Schwarz BioResearch supplies many geneticists and embryologists with nucleic acids, nucleotides, nucleosides, purine and pyrimidine bases — either labeled with $C^{14}$ or $H^3$ or isotopically stable. Consult our catalog for the "nuclein" you need.*



IN APRIL—(1923)—E. N. Harvey[2] tells us that luciferin is far more potent than was previously realized. In 1916 he reported that 1 part of *Cypridina* luciferin in $1.7 \times 10^8$ parts of sea water gave light visible to the unaided eye when mixed with luciferase. He now reports that this estimate can be bettered: one part lucerferin in $4 \times 10^{10}$ parts of sea water!

*Because firefly-catching is more practical than diving for Cypridina, Schwarz BioResearch supplies firefly lanterns to modern students of bioluminescence and for ATP assays. We have received many glowing reports from investigators using both our fireflies and our ATP. Write us for full information and prices.*



IN APRIL—(1943)—Barron and Singer[3] report proof of the role of sulfhydryl groups as enzyme activators. A number of enzymes were treated with inhibitors and then reactivated by addition of glutathione. Enzymes whose activity could be restored by glutathione included pyruvate, maleate, and ketoglutarate oxidases; adenosine triphosphatase; d-amino acid, l-glutamic acid and monoamine oxidases; transaminases; pancreatic lipases; and others. "Since such a large number of enzyme systems contain in their protein moiety -SH groups essential for enzyme activity, the role of glutathione becomes of great importance."

*Today, we are gaining even more understanding of the role of glutathione in enzyme activation; this is true also of cysteine/cystine and their derivatives. Schwarz BioResearch manufactures these, both in stable form and labeled with $S^{35}$. We also provide co-factors such as ATP, DNP, and FAD, that may be required to make the reaction go. Every enzyme chemist should have our catalog and price list.*

1. "J.A.T.": Experimental study of fertilization. Nature *61*:551 (April 5) 1900. 2. Harvey, E. N.: The minimum concentration of luciferin to give a visible luminescence. Science *57*:501 (April 27) 1923. 3. Barron, E. S. G., and Singer, T. P.: Enzyme systems containing active sulfhydryl groups. The role of glutathione. Science *97*:356 (April 16) 1943.

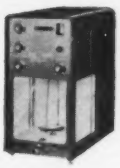
SCHWARZ BIORESEARCH, INC. · Dept. 4G · Mount Vernon, New York

BIOCHEMICALS · RADIOCHEMICALS · PHARMACEUTICALS *for research, for medicine, for industry*

# HARSHAW SCIENTIFIC

# *Spotlights Analytical Balances*

A Balance for every weighing . . . and a Balance that will perform the weighing to the accuracy required in the shortest possible time. Most of our extensive line of domestic and imported balances is illustrated and described in the 32-page Harshaw Scientific Balance Bulletin. Do you have your copy? In addition we will gladly furnish detailed information on particular balances.

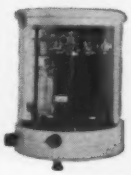


**H-1642**—Balance, Analytical, Ainsworth "Right-A-Weigh," Type SC. Capacity—200 grams. Sensitivity—1/10 mg. Single Pan. *No weight handling.* Features substitution weighing.

Price **$895.00**

**H-2424**—Balance, Analytical, Sartorius "Selecta Rapid" model. Capacity—200 grams. Sensitivity—1/10 mg. Single Pan. *No weight handling.* Weights are an integral part of balance and are added by flick of external knobs.

Price **$890.00**



**H-2400**—Balance, Analytical, Sartorius "Projecta Rapid" Model. Capacity—200 grams. Sensitivity—1/20 mg. Two pan. *No weight handling up to 10 grams* (weights up to 10 grams are built-in).

Price **$645.00**

**H-1823**—Balance, Analytical, Becker Model "AB-2". Two Pan. Dial reading, chainomatic, with notched beam. Capacity—200 grams. Sensitivity—1/20 mg.

Price **$449.00**

**H-1905**—Electrobalance, Cahn, Range Selector Model. For micro weighings quickly and accurately. Maximum range—0-100 mg. with 25 micrograms sensitivity. Four other ranges down to 0-1 mg. with increased sensitivity.

Price **$695.00**

**H-2440**—Balance, Analytical, Voland Model 100N. Capacity—200 grams. Sensitivity—1/10 mg.

Price **$125.00**

These balances are just a few of the many balances making up our complete line including Micro, Semi-Micro, Specific Gravity, etc. Write us regarding your specific need.

## HARSHAW SCIENTIFIC

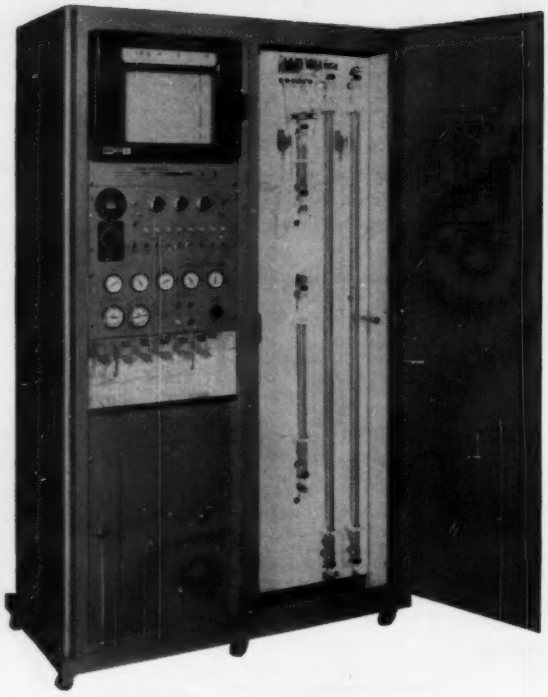
*Division of The Harshaw Chemical Co. • Cleveland 6, Ohio*

*SUPPLYING THE NATION'S LABORATORIES FROM COAST TO COAST*

SALES BRANCHES AND WAREHOUSES
CLEVELAND 6, OHIO — 1945 East 97th Street
CINCINNATI 13, OHIO — 6265 Wiehe Road
DETROIT 28, MICH. — 9240 Hubbell Ave.
HOUSTON 11, TEXAS — 6822 Supply Row
LOS ANGELES 22, CAL. — 2237 So. Garfield Ave.
OAKLAND 1, CAL. — 5321 East 8th Street
PHILADELPHIA 48, PA. — Jackson & Swanson Sts.

SALES OFFICES • Atlanta 5, Ga. • Baton Rouge 6, La. • Buffalo 2, N. Y. • Hastings-On-Hudson 6, N. Y. • Pittsburgh 22, Pa.

# AMINO ACID ANALYZER



## Model K 5000

**FOR SEPARATION, IDENTIFICATION, AND QUANTITATIVE ANALYSIS OF AMINO COMPOUNDS**

***You are cordially invited to visit Booth 50 at the Meeting of the American Society for Clinical Investigation, May 1, 2, 3, 1960. Our display will include Model K-5000 Amino Acid Analyzer; Brice-Phoenix Universal Light Scattering Photometer; Visual and Automatic Recording Differential Refractometers and other items of interest.***

P P I

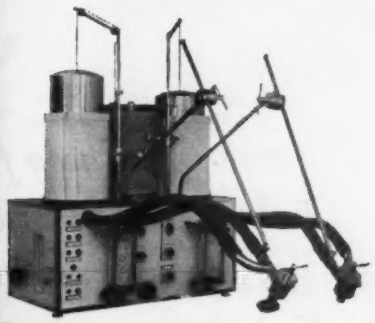
**PHOENIX PRECISION INSTRUMENT COMPANY**

**3803-05 NORTH FIFTH ST., PHILADELPHIA 40, PENNSYLVANIA**

# PULMONARY-FUNCTION EQUIPMENT

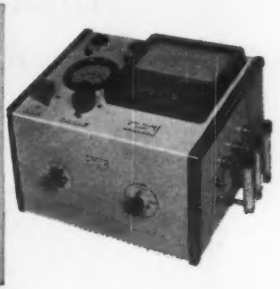
## GODART PULMOTEST

The universal double spirometer, the most compact versatile lung function station for determination of lung volumes and its subdivisions. Maximum breathing capacity, timed vital capacity, and oxygen consumption at rest or in exercise can be determined. The GODART PULMOTEST enables the subject to breathe room air, oxygen being added as it is used, maintaining starting concentration, 3-speed kymograph, motor blower, volume integrator, suitable for closed circuit residual volume determination in conjunction with the PULMO-ANALYSOR.



## GODART PULMO-ANALYSOR

The PULMO-ANALYSOR is a highly accurate thermal conductivity gas analysor which is sensitive to He, $CO_2$ and $O_2$. Usable alone and in conjunction with the GODART PULMOTEST, it can be used to determine residual volume by closed circuit helium method as well as by the open circuit method. Expired gas analysis for $O_2$ and $CO_2$ in mixtures with the highest accuracy, speed and precision.

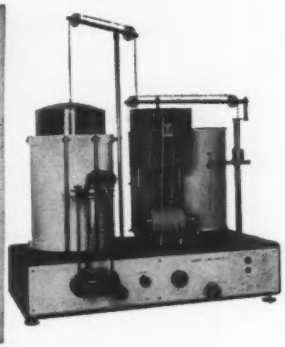


## CAPNOGRAPH

This infra-red gas analysor is used for recording dynamic changes in the level of $CO_2$ in respiration. The response time is rapid (.04 sec.) and is able to follow the respiratory cycle. Alveolar $CO_2$ values are obtainable without recourse to sampling devices. Capacity is highly stable and accurate to 0.1% of its measuring range, compact and ready for immediate use in proper control of artificial respiration, determination of dead space volume, diagnosis of emphysema, chronic hyperventilation.

## GODART COMPLIANCE TEST

To study the elastic properties of the lungs. This equipment records as a kymograph, a loop representing the relation between pressure and volume during respiration. Intra-esophageal pressure changes are detected from a catheter by an electromanometer, volume changes by a precision spirometer and fed into special recorder type kymograph. Supplied with an electromanometer, 3-speed kymograph and all accessories.



**GODART DESIGNED PULMONARY FUNCTION APPARATUS**

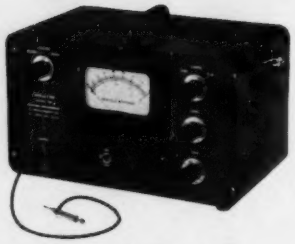
Offices: New York • Utrecht • London

**INSTRUMENTATION ASSOCIATES**

*Distributors of Laboratory and Scientific Specialties*

17 West 60th Street New York 23, N. Y.

*now available for pulmonary function studies in the clinical, research and teaching laboratories*

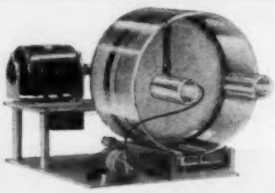


**NITRALYZER 105**

The newest of our continuous reading nitrogen meters, Model 105 is designed for teaching of students and for use in clinics. All routine tests can be performed with this instrument. Nitrogen percentage is read on scale in two ranges, 0-80% and 0-20%. Simple to operate and has a minimum of controls.

**NITRALYZER 300AR**

Our finest nitrogen meter using same sound design as Model 200AR, but incorporating higher sensitivity, 8 ranges including two suppressed zero ranges, a built-in Vacuum Gauge and an output amplifier sufficient to drive most ink-writers DIRECT.



**SERVO-SPIROMETERS 250 and 350**

New concept in volume and flow measuring with high dynamics. These instruments perform most conventional spirometer functions in addition to providing electrical readouts for volumes and flows. The Model 350 can replace the conventional spirometer and the pneumotachograph in many applications; it can drive a mechanical kymograph as well as a galvanometer or a single or double ended recorder.

**MOUSE SPIROMETER 160**

Minute Oxygen Uptake Servo Equipt Spirometer. New highly simplified and economical servo-spirometer especially designed for protracted pulmonary studies, such as oxygen uptake under various conditions on small animals, birds and insects. Yields extremely high volume discrimination at minute pressure levels.

**On display at**
**our**
**Booth No. 6**
**At the Meeting of the**
**American Society for**
**Clinical Investigation**
**Atlantic City, New Jersey**
**May 1–3, 1960**

***CUSTOM ENGINEERING AND DEVELOPMENT CO.***

*2647 LOCUST STREET, ST. LOUIS, 3 MO., Telephone: JEfferson 5-7800*

# New / LOW COST...

# Basic Medical Uptake System

## For THYROID FUNCTION DIAGNOSIS using IODINE-131

Model MTS-175

Complete data available upon request
Ask for bulletin C-175

ATOMIC
*accessories inc.*
*Subsidiary of Baird-Atomic Inc.*

811 WEST MERRICK RD., VALLEY STREAM, NEW YORK

**COMPLETE PACKAGE** . . . Nothing else to buy.

**SIMPLE OPERATION** . . . Can be operated by a technician with a minimum of training.

**HIGH SENSITIVITY** . . . Permits thyroid study with a minimum amount of radioactivity.

**COMPACT & FLEXIBLE** . . . With optimum mobility.

**BASIC** . . . Can be used as the core of a more elaborate isotope laboratory at a later date.

Represented Nationally by ATOMIC ASSOCIATES:
CHICAGO • NEW YORK • PITTSBURGH • PHILADELPHIA
LOS ANGELES • DALLAS • WASHINGTON, D. C. • BOSTON
ATLANTA • DETROIT • SAN FRANCISCO

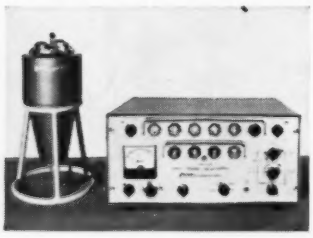
# Fully Automatic, Transistorized AUTO-GAMMA® Spectrometer System

*for counting samples of:*

IODINE$^{131}$ · IRON$^{59}$ · GOLD$^{198}$ · POTASSIUM$^{42}$ · RADIUM DAUGHTERS · COBALT$^{60}$ · CHROMIUM$^{51}$

*. . . and other gamma emitters—*

- up to 100 samples counted automatically
- repeats individual samples or entire loading
- symmetrical geometry provides constant background
- sample number, time and count printed out on paper tape
- integral, differential and wide-window counting modes
- manual model can be automated at any time



The Auto-Gamma Spectrometer System counts and records data from as many as 100 test tube samples. Operation can be maintained on an around-the-clock basis.

The energy spectrum of an isotope can be plotted with the Auto-Gamma Spectrometer by means of the precise Narrow Window setting. Ordinarily, the photopeak is then counted within the Wide Window of the pulse height analyzer to minimize background. This use of the spectrometer optimizes the count-to-background ratio and permits shorter counting periods or lower tracer levels.

Obviously, automatic sample counting is desirable when large numbers of samples are to be counted. It is just as useful, however, for counting small numbers of low activity samples. Blanks and standards can be arranged among the samples for background checks and calibration. The complete series of tubes can then be counted automatically as many times as required to give desired statistical accuracy.

The detector and spectrometer components of the system are available separately for manual operation.

***For complete information write for Bulletin 400.***

**Packard** *Instrument Company, Inc.*

**P. O. BOX 428-J LA GRANGE, ILLINOIS, U. S. A.**

ATLANTA • BOSTON • LOS ANGELES • NEW YORK • PHILADELPHIA
SAN FRANCISCO • WASHINGTON, D.C. • ZURICH, SWITZERLAND

**See us at Booth 25—American Society of Clinical Investigation Show**

LANCASTER PRESS, INC., LANCASTER, PA.